MORPHOLOGICAL EXAMINATION AND IMMUNOSTIMULATION OF THE GASTROINTESTINAL TRACT IN AN *ARTEMIA* MODEL

Sofie Vanmaele

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Promoters:

Prof. dr. ir. Peter Bossier

Prof. dr. Eric Cox

Laboratory of Aquaculture & *Artemia* Reference Center, Department of Animal Production,
Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

Laboratory of Immunology, Faculty of Veterinary Medicine
Ghent University, Ghent, Belgium

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Members of the examination committee:

- Prof. dr. ir. Mieke Uyttendaele, Faculty of Bioscience Engineering, Ghent University
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- Prof. dr. ir. Tom Van de Wiele, Faculty of Bioscience Engineering, Ghent University
- Prof. dr. Patrick Kestemont, Faculty of Sciences, University of Namur
- Em. prof. dr. Patrick Sorgeloos, Faculty of Bioscience Engineering, Ghent University

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LIST OF UNITS & ABBREVIATIONS

%	Percentage
±	Approximately
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
^{0}C	Degree Celcius
$^{-1}$	Per
g	Gram
h	Hour
l	Liter
$m^{-2}s^{-1}$	Square meter per second
min	Minute
ml	Milliliter
mm	Millimeter
nm	Nanometer
s	Second
β	Beta

AMP	Anti-microbial peptides
ANOVA	Analysis of variance
bp	base pairs
cDNA	Complementary deoxyribonucleic acid
DAH	Day after hatching
DNA	Deoxyribonucleic acid
Dscam	Down syndrome cell adhesion molecule
Esod	Extracellular superoxide dismutase
FAO	Food and Agricultural Organization of United Nations
FASW	Filtered and autoclaved seawater
GI	Gastrointestinal
H6	*Vibrio* spp strain HABRA 6 isolated from *L. vannamei*
Hsp70	Heat shock protein 70
Lgbp	lipopolysaccharide and β-1, 3-glucan binding protein
LGBP	Lipopolysaccharide and β-1,3/1,6-glucan binding protein
LPS	Lipopolysaccharide
LVS 3	*Aeromonas hydrophila* Strain 3 isolated by Laurent Verschuere
MA	Marine agar 2216
MAMP	Micro-organism associated molecular pattern

Masq	Masquarade-like protein
MB	Marine broth 2216
mRNA	Messenger ribonucleic acid
n	Number of replicates
OD	Optical density
p	Statistical p-value obtained
PAMP	Pathogen associated molecular pattern
PCR	Polymerase Chain Reaction
Pero	Peroxinectin
PGN	Peptidoglycans
PGRP	Peptidoglycan recognition protein
pH	Measure of the acidity of solution
PI	Propidium iodide
ProPO	Prophenoloxidase
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per minute
RPS	Relative percentage survival
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
SG	SYBR Green
Tgase	Trangslutaminase
TLR	Toll-like receptor

CHAPTER 1

INTRODUCTION

CHAPTER 1: Introduction

1.1. *Artemia franciscana* & the global aquaculture industry

1.1.1. The global aquaculture industry

Ever since fish have taken up an essential place in healthier human diets, aquaculture has become a very important part of human food production (Figure 1.1). Aquaculture has been standard practice over many centuries in some countries and continents, especially Asia. When compared to the terrestrial food production sector the global aquaculture sector is very young, but it has known a rapid expansion the last 50 years (FAO, 2012). The latest FAO report shows that both capture fisheries and aquaculture supply worldwide about 158 million tonnes of fish for food consumption as for secondary purposes (Figure 1.2) (FAO, 2014).

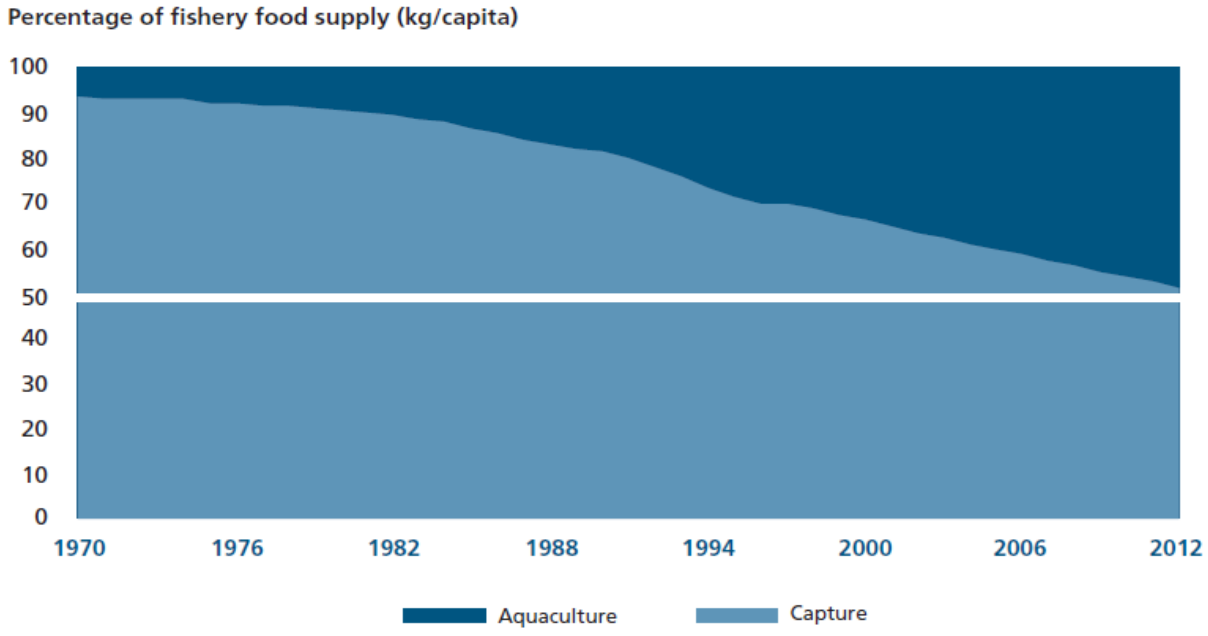

Figure 1.1: Relative contribution of aquaculture and capture fisheries to food fish consumption (Source: FAO, 2014)

Aquaculture includes the farming of all aquatic organisms such as fish, shellfish and even plants. It takes into account the cultivation of marine, brackish and freshwater species and can range from land-based to open-ocean production. Currently, over 600 aquatic species (Figure

1.3) are being grown in captivity. This culturing of these different marine, brackish and freshwater species is performed in a huge variety of culturing systems and technological advances (FAO, 2012). Aquaculture does not only provide cultured animals destined for the food market. Hatchery-produced seed is also widely used for restocking purposes of capture fisheries, especially in inland waters. In 2012, aquaculture production was estimated at a total value of US$ 144.4 billion, corresponding to 90.4 million tonnes, when excluding non-food products. In comparison with 2001, this was only 34.6 million tonnes. Cultured species vary greatly between marine, brackish and freshwater aquaculture (FAO, 2014). Crustaceans play an important role in brackish water culturing (57.2%, 2.7 million tonnes) compared to marine crustaceans (3.8%) and freshwater crustaceans (6.4%) (FAO, 2012). In the world aquaculture production they compose 9.7% of total production or 6.4 million tonnes by volume but around 22.4% by value or US$ 30.9 billion (FAO, 2014).

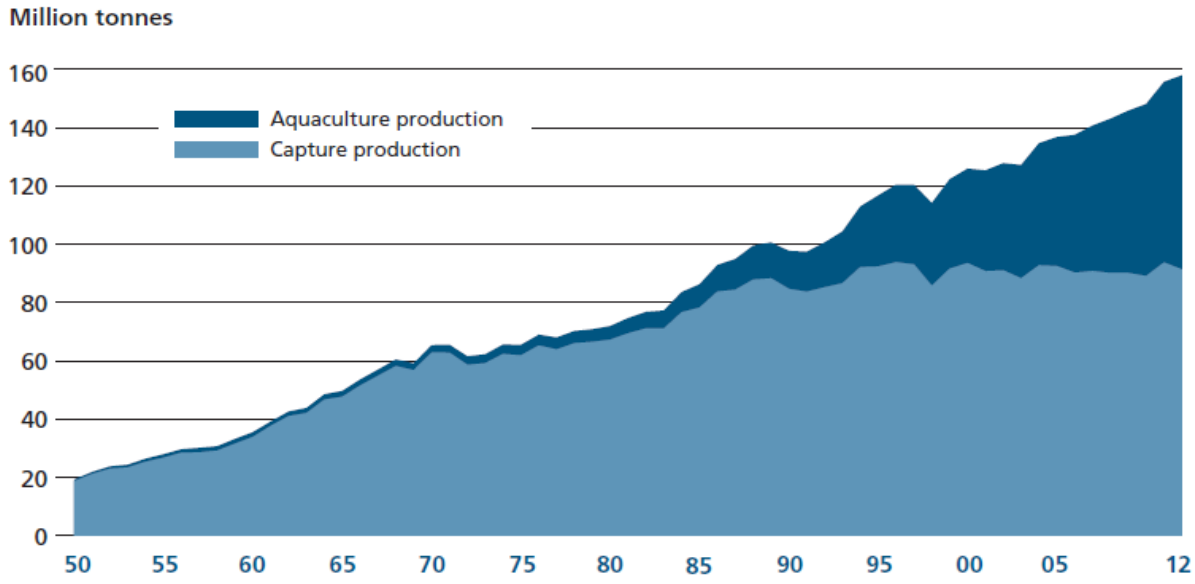

Figure 1.2: World aquaculture production compared to capture fisheries from 1950 to 2012 (Source: FAO, 2014)

The FAO report of 2012 projected that a continuing increase to around 80 million tonnes will be needed by 2050 to satisfy the current level of per capita fish consumption. This increase will have to come from aquaculture since the last years it has become increasingly clear that capture fisheries cannot keep up with this rising trend. Wild captures have been stagnant at around 90 million tonnes since 2001 and the majority of the main fishing areas have reached their maximum potential some of which are dangerously overfished.

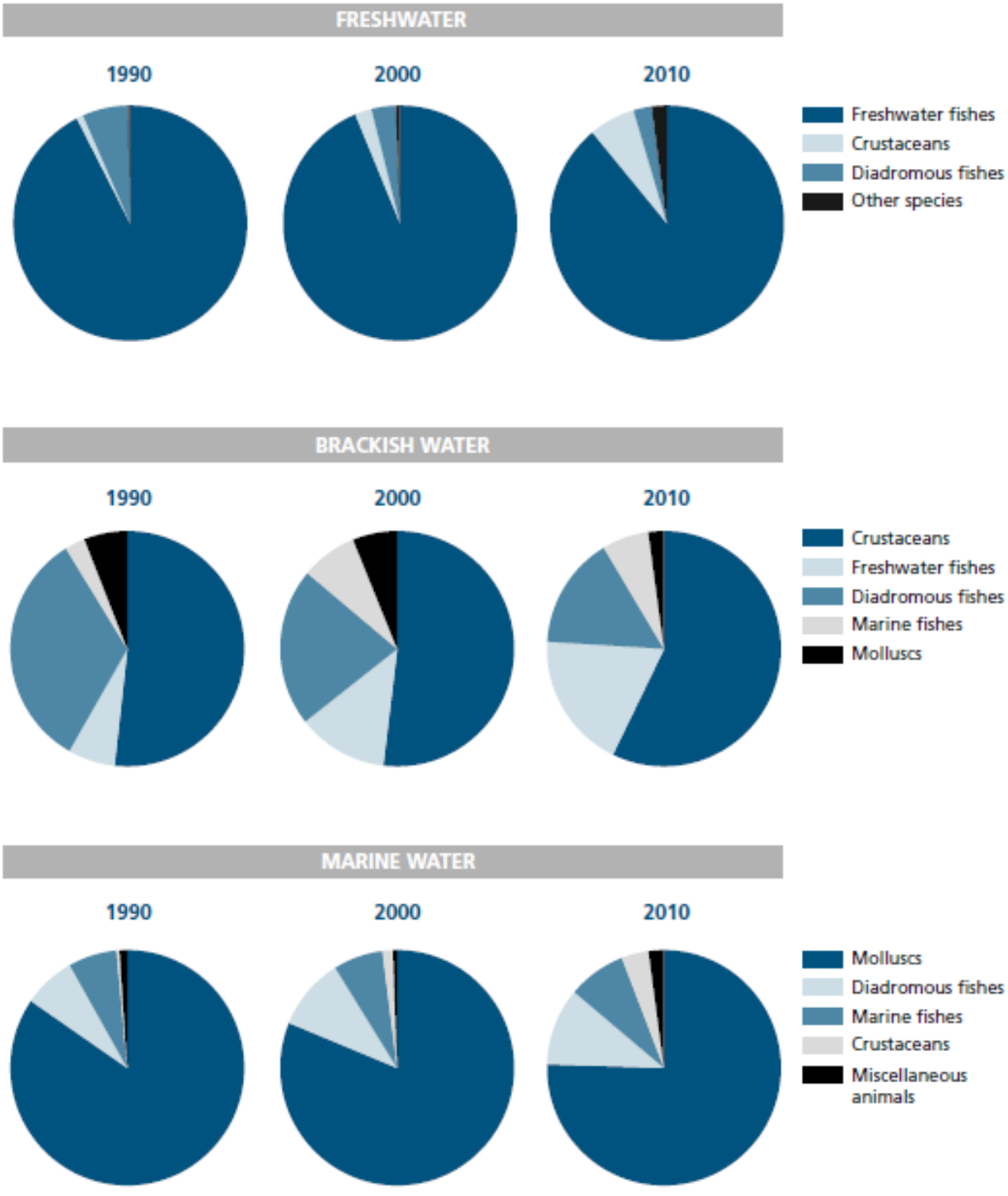

Figure 1.3: World aquaculture production composition by culture environment (Source: FAO, 2012)

1.1.2. The importance of *Artemia franciscana* in aquaculture production

Artemia franciscana is a small branchiopod crustacean found almost worldwide in natural saline lakes and solar salt works (Figure 1.4). They are used worldwide as live feed in aquaculture production. It has also been used as a model organism for host-microbial studies among others (section 1.5). They are unique organisms that can produce dormant cysts or encapsulated embryos, called cysts, when conditions are unfavorable (Léger *et al*., 1986). These cysts can be stored during a long period of time in a dry state, making them readily available on the market (Sorgeloos, 1986). When brought back into favorable conditions, the cysts, if out of diapause, will hatch within 24 h and can be used as a live feed for culturing other species or for scientific purposes.

Figure 1.4: Nauplius of *Artemia* from Great Salt Lake

Artemia franciscana is a small-sized organism with a short generation time of 2-3weeks and it takes them, under optimal conditions, about 8 days to reach adult stage (Figure 1.5). They are extremely osmotolerant animals that can survive in a broad range of salinities from 5g l^{-1} to around 200g l^{-1} yet they can also be found in brackish and supersaturated waters of 340g l^{-1}. They are not only tolerant of a high range of salinities but also of variable temperatures (6-35°C), ionic composition and a pH tolerance from neutral to highly alkaline

(Van Stappen, 1996). They can easily be cultured in high densities on a small scale, using a very simple standardised culture system.

The presence of infectious pathogens can cause massive mortalities in *Artemia* cultures and they can be a vector in disease transmission, especially concerning *Vibrio* sp. (Gomez-gil *et al*., 1994; Muroga *et al*., 1994; Verdonck *et al*., 1994). To avoid this the use of prophylactic and environmentally friendly approaches is being researched by inducing protection against infectious diseases in *Artemia* before being added to other aquaculture systems (Marques *et al*., 2006).

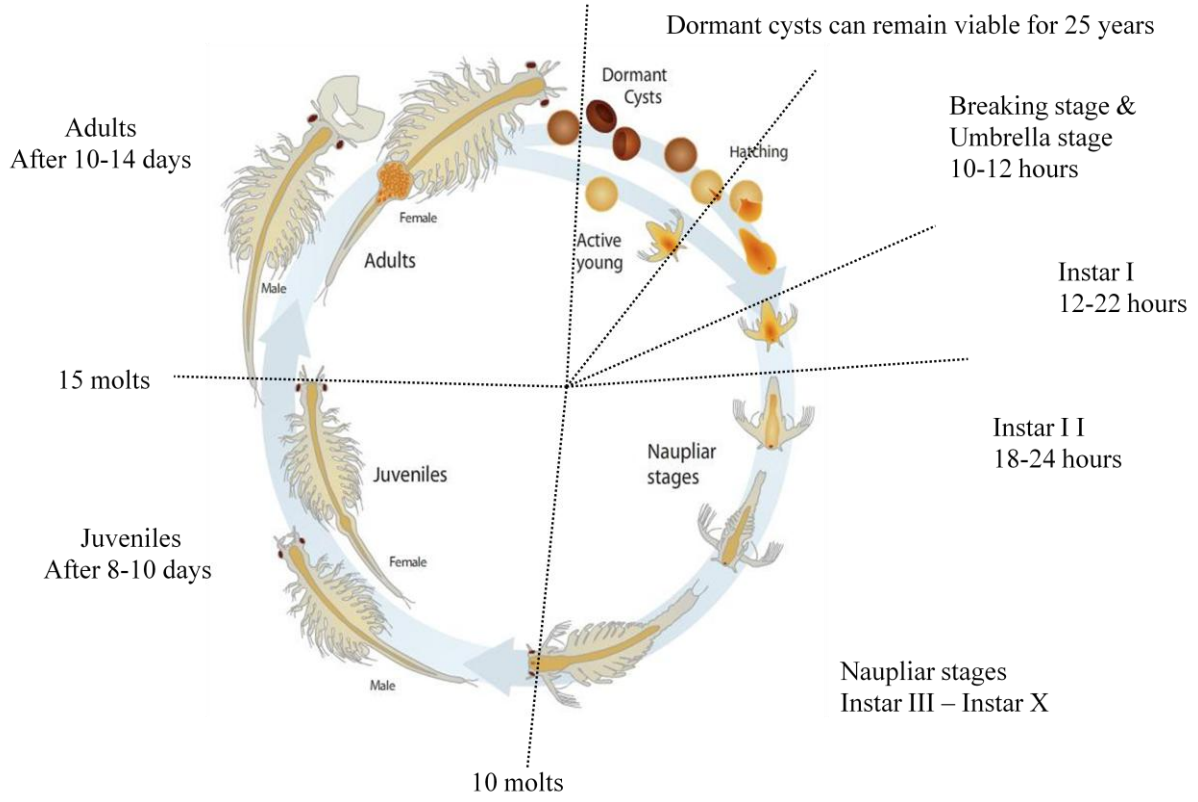

Figure 1.5: Life cycle of *Artemia franciscana*
(Adapted from: University of Utah, Genetic Science Learning Center)

1.2. Threats & diseases in aquaculture

Several threats are related to the current ways of culturing marine animals, for example the introduction of new species, the risk of escapees, eutrophication and damage to the ecosystem. Nonetheless, sustainable growth in aquaculture is mainly hampered by increasing

incidences of disease causing major economic losses (Bachère, 2003) and the correlated misuse of antibiotics. Several species belonging to the *Vibrio* genus, including *V. anguillarum, V. alginolyticus, V. harveyi, V. campbellii, V. splendidus and V. coraliilyticus*, have become nightmares in the aquaculture industry by causing vibriosis (Toranzo & Barja, 1990; Aguirre-Guzman *et al*., 2004). Vibriosis is a disease which causes several billion US$ losses each year. It is a disease that occurs rapidly, induces weak muscular movements, red discoloration and results in high mortality in shrimp, fish and mollusk cultures (Adams & Boopathy, 2013). For bacteria to infect their host several steps are involved. Firstly they have to enter the host, after which establishment and multiplication occurs and damage is done to the host tissues followed by death. The pathogenesis of the closely related *Vibrio harveyi* and *V. campbellii* has not been fully elucidated yet, but it is known that *V. anguillarum* goes through the skin and *V. campbellii* causes damage to the intestinal epithelium (Ruwandeepika *et al*., 2012; Austin & Zhang, 2006). Biofilm production and motility are important for entering the host animals (Ruwandeepika *et al*., 2012). Extracellular products have been identified to be important to virulence of *V. harveyi* towards tiger prawn together with proteases, phospholipase and haemolysins. On the contrary, in infection of *Artemia franciscana* nauplii proteases and phosopholipases play a significant role and not lipase, gelatinase or haemolytic activity (Soto-Rodriguez *et al*. 2003). Some strains of *V. harveyi* and *V. campbellii* are luminescent and infections by such strains are characterised by the fact that the infected animals glow in the dark (Thran *et al*., 2013).

1.2.1. Antibiotics for controlling disease

Most of the (super) intensive culturing systems are related with stressful conditions such as overcrowding, high temperatures and improper waste removal, especially in the invertebrate farming. The presence of waste products coming from uneaten feed and excreta promotes proliferation of opportunistic bacteria that will infect the live food and the larvae under these poor environmental conditions (Almeida *et al.*, 2009; Banerjee *et al*., 2014). To prevent the colonisation of the cultured animals by harmful pathogens, antibiotics have often been used in the past. Antibiotics are natural or synthetic compounds that kill bacteria or restrain their growth. The occurrence of antibiotic resistant pathogens renders antibiotics ineffective as treatment option and therefore their application in aquaculture has been scaled back (Defoirdt *et al*., 2007; ECDC, 2014). Introduced in the culture systems they can ‘spill’ also in the surrounding environment where they can be ingested by the wildlife such as fish

and shellfish. Studies showed that antibiotics can appear in sediments of the natural environment as residues where they can pilot alterations of the existing microbiota, favouring the development of antibiotic resistant strains (Defoirdt *et al*., 2007; Banerjee *et al*., 2014). Since bacterial species can double their population relatively quickly, some in terms of hours, they also possess great powers of adaptation through mutation in order to survive in the environment. Resistance is also being facilitated by horizontal gene transfer between aquatic and terrestrial bacteria, by contact between contaminated sea water and freshwater. The presence of residual antibiotics in commercial fish and shellfish products (Marshall & Levy, 2011) can possibly lead to allergy and toxicity in humans (Alderman & Hastings, 1998; Cabello, 2006; FAO, 2012). To obtain a more sustainable production, other strategies to control diseases are needed. Other approaches that have been established in aquaculture are e.g. improving pond water quality, host health, vaccines, phage therapy, macrophyte-based treatments, immunostimulants and probiotics (Marques *et al*., 2006; Defoirdt *et al*., 2007; Banerjee *et al*., 2014; Miest & Hoole, 2014).

1.3. Immune defences of invertebrates

1.3.1. The innate immune system

Although the immune system of the 45 000 vertebrates and over 10 million invertebrates greatly differs, they do share a common ancestor from hundreds of millions of years ago, leading to a shared innate immune system (Ellis *et al.*, 2011). Nonetheless, the divergence during evolution has contributed to changes, e.g. the Toll pathway of the fruit fly (*Drosophila melanogaster*) is also involved in the development of the animal, not only in immune defence (Loker *et al*., 2004). Now, the immune system is not only responsible for recognising harmful pathogens but also commensal microbiota colonising the host animals (Chu & Mazmanian, 2013). It is very important to understand the immune responses of commercially important invertebrate species in aquaculture as diseases cause such high economic losses. The added difficulty is that those invertebrates often have multiple life-history stages separated by complete metamorphosis. Until now, it remains unclear how host–microbe interactions change in the light of these different life-history stages of the animals (Thomas & Rudolf, 2010).

The major players of the specific or acquired immunity found in vertebrates are the lymphocytes or white blood cells with a great variety of receptors like immunoglobulins and T-cell receptors. These are capable of recognising any pathogen an animal might come across during its lifetime (Ghosh *et al.,* 2011). For this specific immune defence system to work most efficiently, it takes a couple of days or weeks. The specific immune defences that can be found in mammalians and vertebrates are not present in invertebrates. On the contrary, their innate immune response doesn't require previous exposure to a pathogen, but it offers immediate and effective protection against a wide variety of pathogens, a first emergency response so to say. Yet the protection is most of the times short-lived, there is no memory as present in vertebrates (Beutler, 2004), although some studies do speak about more efficient immune responses at a second exposure by the same type of pathogen (Nyholm & Graf, 2012).

The innate immune response of crustaceans consists of several receptors and pathways which can greatly differ between different species (Kimbrell & Beutler, 2001; Vazquez *et al*., 2009). Three overall defence systems are present: a physical barrier namely the exoskeleton and alimentary tract, cellular defence mechanisms and humoral defence reactions, responsible for the production of immune proteins e.g. antimicrobial peptides and proteinase inhibitors. Cellular responses as phagocytosis, nodulation and encapsulation are triggered by compounds present in the hemolymph (Tassanakajon *et al.,* 2013).. The prophenoloxidase system, phagocytosis and encapsulation are all immune pathways that will be activated upon the recognition of foreign material present in this hemolymph after they have passed the first barrier, namely the carapace. These humoral responses are induced and secreted from the hemocytes (Hauton, 2012; Vazquez *et al*., 2009). Hemocytes are responsible for wound repair and blood coagulation to prevent the pathogen from entering the hemocoel (Cerenius & Söderhäll, 2011). When pathogens gain access, the blood cells will execute phagocytosis by encapsulation after which the nodule formation occurs and the pathogen is isolated in the host tissue. Three classes of hemocytes are present in crustaceans: hyaline cells are responsible for phagocytosis, semigranular cells for encapsulation and granular cells produce antimicrobial peptides (Table 1.1). Degranulation of these semigranular and granular hemocytes releases a battery of potent immune effector molecules as for example the proPO cascade (Lin & Söderhäll, 2015).

Invaders of the host system are recognised by pattern-recognition receptors (PRRs). Some of the most well-known PRRs are Toll-like receptors (TLRs), peptidoglycan recognition proteins (PGRPs), lectins and lipopolysaccharide and β-1,3-glucan binding protein (LGBP). PRRs recognise the pathogen-associated molecular patterns (PAMPs) from bacteria, fungi or parasite surfaces for example, β-1,3/1,6-glucans from fungi, peptidoglycans (PGN) or lipopolysaccharide (LPS) from bacteria and double stranded RNA of parasites and trigger signalling pathways (Cerenius *et al.*, 2010). These PAMPS are highly conserved regions present in the cell wall of these micro-organisms. Yet, these PAMPs are not only specific to pathogens, but are also present in beneficial or benign microorganisms, therefore also referred to as microorganism-associated molecular patterns or MAMPs (Cerenius *et al.*, 2010).

Table 1.1: Effector defence mechanisms in crustaceans (Source: Vazquez *et al*., 2009)

Defense mechanisms	Cellular population involved	Target
ProPO	Semigranulocytes, hemocytes with big refractile granules	Bacteria and fungi
Antimicrobial proteins	Hemocytes with granules	Bacteria and fungi
Phagocytosis	Hyalinocytes, semigranulocytes	Bacteria and micro-organisms <10 µm
Encapsulation	Semigranulocytes hemocytes with big refractile granules	Fungal spore and yeast, organisms >10 µm
Lectins	Hyalinocytes, semigranulocytes and hemocytes with refractile granules	Distinguish between the self and non-self particles, inducing agglutination and phagocytosis
Clottable protein	Clottable proteins from hemocytes	Bacteria and fungi

Toll-like receptors are activated by Gram-negative bacteria, which will induce the production of proteases leading to the activation of the Toll receptor ligand Spätzle. These cause the dimerization of the Toll receptor and phosphorylation of the intracellular Toll/interleukin-1 recepter (TIR) domain. Activation of the Toll-like receptor will result in the production of antimicrobial peptides (AMPs) (Hauton, 2012). Another important PRR are the Down Syndrome Cell Adhesion Molecule (DSCAM) receptors. DSCAM receptors are cell membrane molecules with a cytoplasmic tail and an extracellular region. mRNA transcripts of DSCAM have been found in the fat bodies of insects (Hauton, 2012). This extracellular region shows a very high level of variability. This variability has been put forward as a possible explanation for the observed phenomenon of specificity within the innate immune system in crustaceans (Chou *et al*., 2011; Watthanasurorot *et al*., 2011). The phenoloxidase (PO) cascade is a complex cascade triggered by tiny amounts of MAMPs, e.g. peptidoglycans or β-glucans. Serine proteinases will perform the proteolysis of proPO into catalytically active

PO. Active PO oxidises phenols into quinones, regulating the melanin production. Melanin production is very precise and only at the injury or on the pathogen in order not to harm the host tissue (Cerenius *et al.*, 2010; Rowley & Powell, 2007; Vazquez *et al*., 2009). This cascade can be compared to the complement system in vertebrates, although here the end product is melanin. Finally, unwanted cells are removed from the system by means of apoptosis, a genetically regulated process for elimination of damaged or harmful cells (Tassanakajon *et al*., 2013).

The activation of proteolytic cascades will result in clotting via the release of transglutaminase from the hemocytes which initiates the polymerization of the clotting protein and melanisation cascades (phenoloxidase system) in the hemolymph (Beutler 2004). Both mechanisms are working together and are well coordinated (Rowley & Powell, 2007). The coagulation system is responsible for prevention of blood or fluid loss after injury, since invertebrates have an open circulatory system (Cerenius & Söderhäll, 2011). In crustaceans, two components can be found: a transglutaminase released from the blood cells and a circulating plasma protein. This is a homodimer protein, rapidly polymerised by the transglutaminase. The horseshoe crab clotting system however, is a proteolytic cascade, initiated by PRRs (Cerenius *et al*, 2010). These invertebrate coagulation factors are not present in vertebrates. Humoral and cellular immune responses are very much intertwined when the immune system is being stimulated. The production of anti-microbial peptides is the outcome from certain humoral response. These AMPs are responsible for the lysing of cells, together with reactive oxygen species (ROS). In shrimp, some well-known AMPs are penaeidins and crustins (Rowley & Powell, 2007; Ghosh *et al*., 2011; Nyholm & Graf, 2012). These PRRs, anti-microbial peptides (AMPs), oxygen radicals and melanisation pathways are just a couple of the things involved in innate immune responses in invertebrates, some of which are shown in Figure 1.6.

To determine levels of these cellular and humoral parameters in crustaceans, several procedures are in place. For cellular activity measurements a hemocyte count can be performed, although for the case of *Artemia* this is complicated. Radical oxygen intermediates (ROIs) and measurements of prophenoloxidase and phenoloxidase activity are more straightforward as immunological markers. An indicative parameter of the humoral stimulation is the plasma protein concentration (Rodriguez & Le Moullac, 2000).

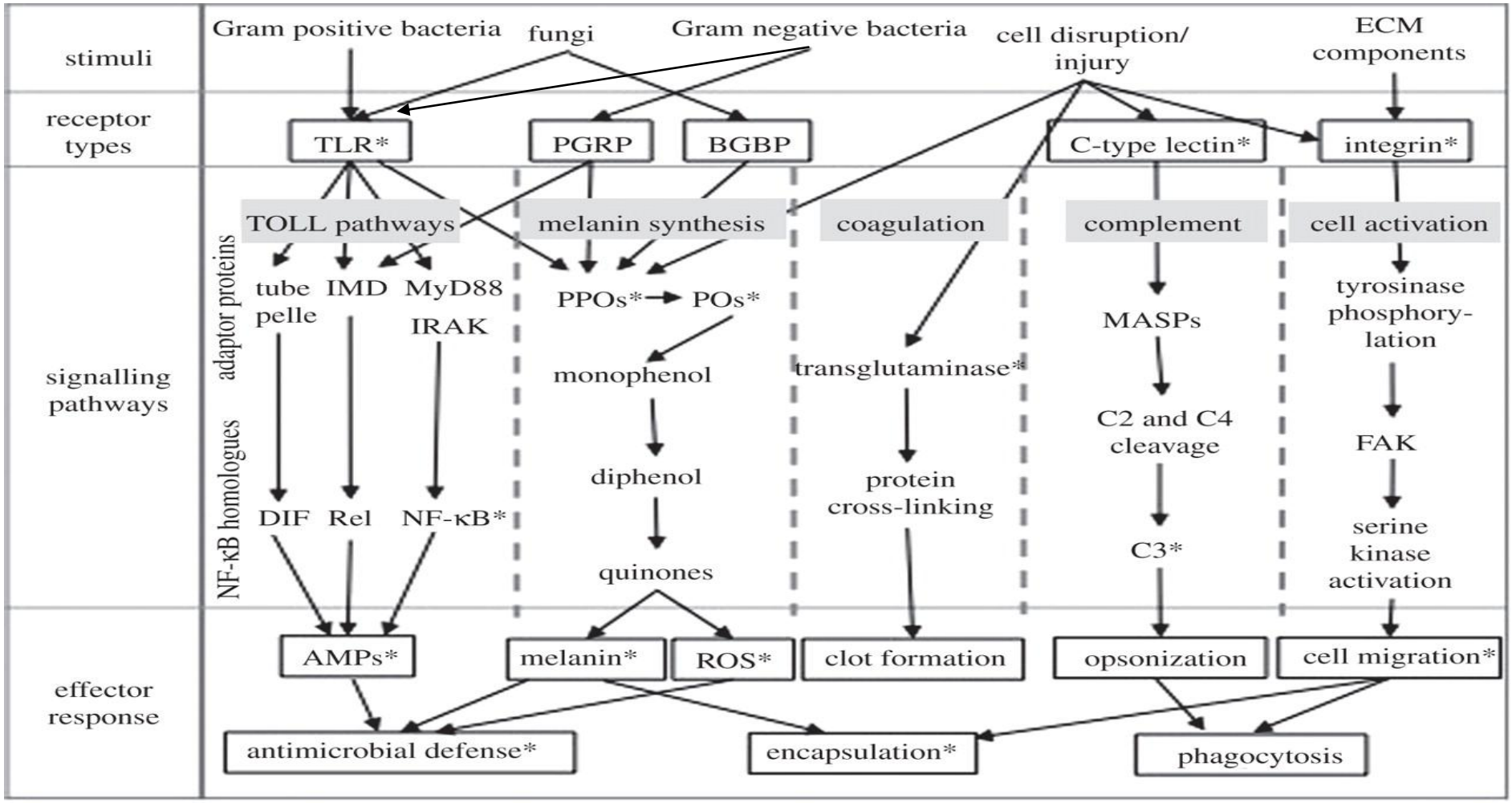

Figure 1.6: A schematic overview of the immune system of invertebrates. Legend: TLR: TOLL-like receptor; PGRP: peptidoglycan recognition protein; BGBP: beta-glucan binding protein; IMD: immunodeficiency; MyD88: myeloid differentiation primary-response protein 88, PPOs: prophenoloxidases; Pos: phenoloxidases; MASPs: mannose binding lectin-associated serine proteases; FAK: focal adhesion kinases; AMPs: antimicrobial peptides; ROS: reactive oxygen species (Adapted from: Palmer & Traylor-Knowles, 2012)

1.3.2. Immune-related genes

The *Artemia franciscana* genome size has been estimated in 2013 by De Vos and colleagues using flow cytometry. The genome size of a haploid adult proved to be 0.93 10^9 bp, which is a small genome size for a crustacean (De Vos *et al*., 2013). Shrimps for example have a genome size of approximately 2 10^9 bp (Hu *et al*., 2014). The genome sequencing of *Artemia franciscana* is currently being performed (Ghent University non-disclosed information at the Lab Aquaculture & *Artemia* Reference Center). As mentioned before, immunostimulants are being used as an alternative to antibiotics in the fight against disease in aquaculture. Up to now, not much is known about how these compounds influence or affect the gene expression of exposed *Artemia* nauplii. Therefore, eight genes that are correlated with the innate immune response of invertebrates were investigated in this study: prophenoloxidase (*proPO*), transglutaminase (*tgase*), heat shock protein 70 (*hsp70*), masquarade like protein (*masq*), extracellular superoxide dismutase (e*SOD*), peroxinectine (*pero*), lipopolysaccharide and β-1, 3-glucan binding protein (*lgbp*) and Down syndrome cell adhesion molecule (*dscam*).

Tgase and ProPO are known indicators for the immune response that helps fight infection and Hsp70, which is an immune modulator and can be upregulated after infection (Figure 1.7). Transglutaminases (Tgases) are enzymes involved in the blood clotting system and in the molting process to prevent blood loss and help obstruct pathogens from attacking the wound (Liu *et al*., 2007; Maningas *et al*., 2013). They are produced by the hemocytes of the invertebrate as a response to tissue damage (Wang *et al*., 2001). The prophenoloxidase (proPO) activating system is important in the crustacean immune system as it is one of the major immune defence mechanisms against several pathogens for invertebrate animals (Cerenius and Söderhäll, 2004). Heat shock proteins are involved in the folding and unfolding of other proteins and are induced when the host is exposed to high temperatures or other stress factors. Hsp70 can be both intracellular providing cytoprotection through protein folding, as extracellular facilitating immune responses (Baruah, 2012). The accumulation of Hsp70 is related to a higher tolerance for disease. They are known to be molecular chaperones but seem also be able to mediate humoral and cellular innate immune responses through Toll-like receptors (Sung & MacRae, 2011).

The Down syndrome cell adhesion molecules (Dscam) are receptors present in crustaceans and are involved in neural development (Hauton, 2012; Watthanasurorot *et al*., 2011). They play an essential role in neuronal wiring and the innate immune system of crustaceans. A previous study with the water flea (*Daphnia magna*) showed that the Dscam molecule is a single-locus gene with an extensive diversification (Brites *et al*., 2008). Dscam can produce variants with specific binding capabilities, specifically for a certain immune challenge or infection. These many isoforms could offer a possible explanation for the observed phenomenon of immune priming in crustaceans (Rowley & Pope, 2012).

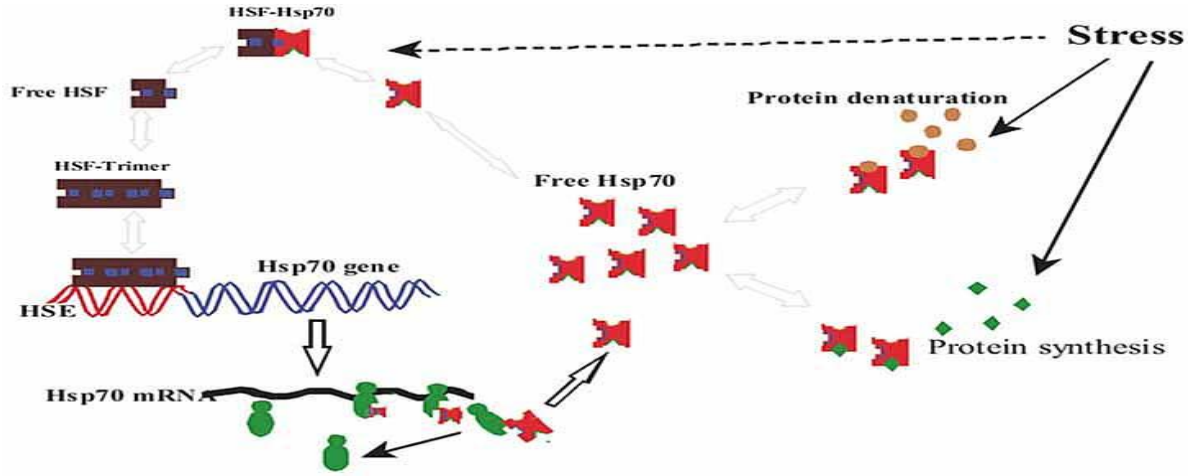

Figure 1.7: Hsp70 induction and regulation: cellular changes in proteins caused by stress are captured by Hsp70, which induces a dissociation of the HSF-Hsp70 complex and triggers gene transcription of Hsp70. Hsf, Heat shock factor; Hsp70, Heat shock protein 70 (Source: Liu *et al*., 2006)

The Masquarade-like protein (Masq*)* has cell adhesion properties and can bind Gram-negative bacteria and yeast cell walls, after which the masquarade-like protein is processed by a proteolytic enzyme and plays a crucial role as an innate immunity protein (Cerenius & Söderhall, 2004). Lipopolysaccharide and β-1, 3-glucan binding protein (LGBP) is a pattern recognition receptor for lipopolysaccharides and β-1, 3-glucans, the latter being present in Gram-negative bacteria and fungi. They are considered to be microorganism-associated molecular patterns (MAMP's) (Yu *et al*., 2001). Peroxinectin (Pero) is a cell adhesion protein functioning as a peroxidase and an opsonin. The formation of peroxinectin is activated by lipopolysaccharides and β-glucans (Johansson *et al*., 1999). It activates the prophenoloxidase cascade and results in phagocytosis or encapsulation (Hauton, 2012). Extracellular superoxide dismutase (eSOD) plays a major role as an antioxidant defence mechanism. It is an enzyme which turns superoxide (O_2^-) into oxygen (O_2) and hydrogen peroxide (H_2O_2) (Holmblad &

Söderhall, 1999). Superoxide is toxic and is produced by the phagocytes to kill pathogens. Meng and colleagues (2011) showed that induction of the gene expression increased resistance of the Chinese mitten crab (*Eriocheir sinensis*) against infection by *Spiroplasma eriocheiris.*

1.4. Immunostimulation, priming & β-glucans

1.4.1. Immunostimulants in aquaculture

Immunostimulants are substances that stimulate the immune system. These refer to any chemical or natural compound that triggers the non-specific defence system to render animals more resistant to infection by a harmful micro-organism (Smith *et al*., 2003). These substances can be drugs or nutrients and are often classified in groups, e.g. bacterial, algae- or animal-derived, etc. Several immunostimulants are currently being used in aquaculture, for example alginates, glucans, chitosans, bacterial cell wall compounds and even extracts of medicinal plants as ginger (*Zingiber officinale*) or mistletoe (*Viscum album*) (Ringø *et al*., 2012; Ganguly *et al*., 2010; Bricknell & Dalmo, 2005; Dügenci *et al*., 2003). In terrestrial animals the benefits of phytochemicals such as alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids and essential oils have already been demonstrated (Chakraborty & Hancz, 2011). Therefore the use of these plant-derived compounds coming from fruits, vegetables, beans and cereals has now been applied in aquaculture as well (Chakraborty & Hancz, 2011). Another group of frequently applied as immunostimulant in aquaculture are prebiotics, e.g. as fructooligosaccharide, mannanoligosaccharide and inulin (Song *et al*., 2014).

Different immunostimulants are already widely used in intensive shrimp culture; but there remains a reasonable degree of doubt about their efficacy. This is not only because there is a lack of unequivocal evidence for their beneficial effects in reducing susceptibility to diseases or infection, but also because the use of such compounds over a prolonged period might be detrimental to the host (Figure 1.8) (Smith *et al*., 2003). Among various kinds of immunostimulating substances, β-glucans have proven potent, valuable and promising compounds for improving the immune status of aquatic organisms (Robertsen *et al*., 1994; deBaulny *et al*., 1996).

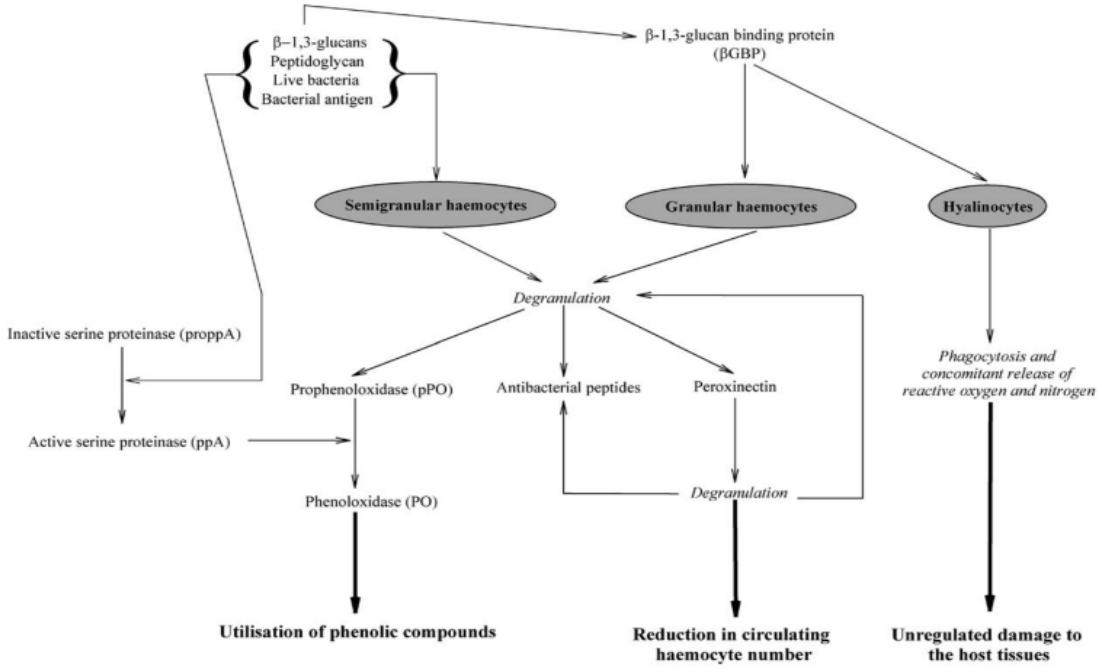

Figure 1.8: Potential consequences of overstimulation of the crustacean immune system with immunostimulants (Source: Smith *et al*., 2003)

1.4.2. β-glucans as immunostimulants

β-glucans are one of the most current used products for immunostimulation in aquaculture. The most common source is the cell wall of baker's yeast (*Saccharomyces cerevisiae*) providing both the β-1,3-glucans and the β-1,6-glucans (Meena *et al*., 2013). Even so other sources of β-glucans are available as well, for example seaweed (*Laminaria* sp.), mushrooms like Shiitake (*Lentinus edodes*), Maitake (*Grifola frondosa*) and *Schizophyllum commune*. The difference between these β-glucans derived from other sources and β-1,3/1,6-glucans are the differences in their structure which triggers different innate immune reactions (Meena *et al*., 2013).

β-glucans are known for triggering the innate immune system in crustacean species. Structurally, β-glucans are polysaccharides consisting of a glucose residue jointed by a beta linkage (Chen & Seviour, 2007). MacroGard is among one of the better known immune modulating β-1.3/1.6-glucans, produced from a specially selected strain of the brewer's yeast *Saccharomyces cerevisiae* (Siwicki *et al*., 1998; Pal *et al*., 2007). It has been widely used in immune therapy of both aquatic and terrestrial animals for almost 25 years and the efficacy of MacroGard under field conditions has been well documented (Sych *et al.*, 2013*;* Miest *et al*.,

201; Ringø *et al*., 2012; Siwicki *et al.*, 2010; Siwicki *et al.*, 2009; Bagni *et al*., 2005; Siwicki *et al.*, 2004; Supamattaya *et al*., 2000).

In invertebrates, β-glucans induce two types of humoral responses: coagulation and anti-microbial peptide production (Figure 1.9). In contrast with vertebrates, where soluble and insoluble β-glucans are recognised exclusively via a number of cell surface receptors, the recognition of β-glucans in invertebrates appears to occur primarily in the haemolymph via a number of completely unrelated proteins. β-glucans are recognised in invertebrates by pattern recognition receptors, such as factor G, Toll-like receptors (TLRs) and β-1,3-glucan binding protein (BGBP) (Figure 1.8). This in turn induces a series of sequential proteolysis events as mentioned before. In Indian white shrimp fed with marine yeast cells, glucans showed to significantly improve survival against White spot syndrome (WSSV) when they were administered once every seven days (Sajeevan *et al.* 2009). Studies from Marques *et al.* (2005) and Soltanian (2007) showed that a daily addition of small amounts of yeast and/or pure glucans provide *Artemia franciscana* with protection against *Vibrio campbellii.*

1.4.3. Priming in invertebrates

Priming is the induction of increased resistance in the host organism after a first exposure to an immunomodulating compound or micro-organism (Schmid-Hempel, 2005). Several studies showed some form of acquired immunity by previous contact with pathogens or biological polymers (Kurtz & Franz, 2003; Hauton & Smith, 2007). This mechanism has already been demonstrated in plants (Paulert *et al*., 2010) and in invertebrates where a high degree of specificity to microbial compounds has been discovered (Schulenburg *et al*., 2007). An attempt has been made to identify the underlying mechanisms involved in priming using the *Drosophila-Streptococcus* pneumonia host-pathogen model (Hauton & Smith, 2007). Results indicate that the phagocytes are responsible for immunity and that their activity is primed by the exposure to an initial sub lethal dose. However, it is not clear from these data whether this is due to increased functioning of existing phagocytes, or to some other aspects of the immune system that have not been characterised yet (i.e. the proPO-system). In the woodlouse (*Porcellio scaber*), hemocytes showed increased phagocytosis of a previously encountered bacterial strain compared to other bacteria after priming with heat-killed bacteria (Roth & Kurtz, 2009).

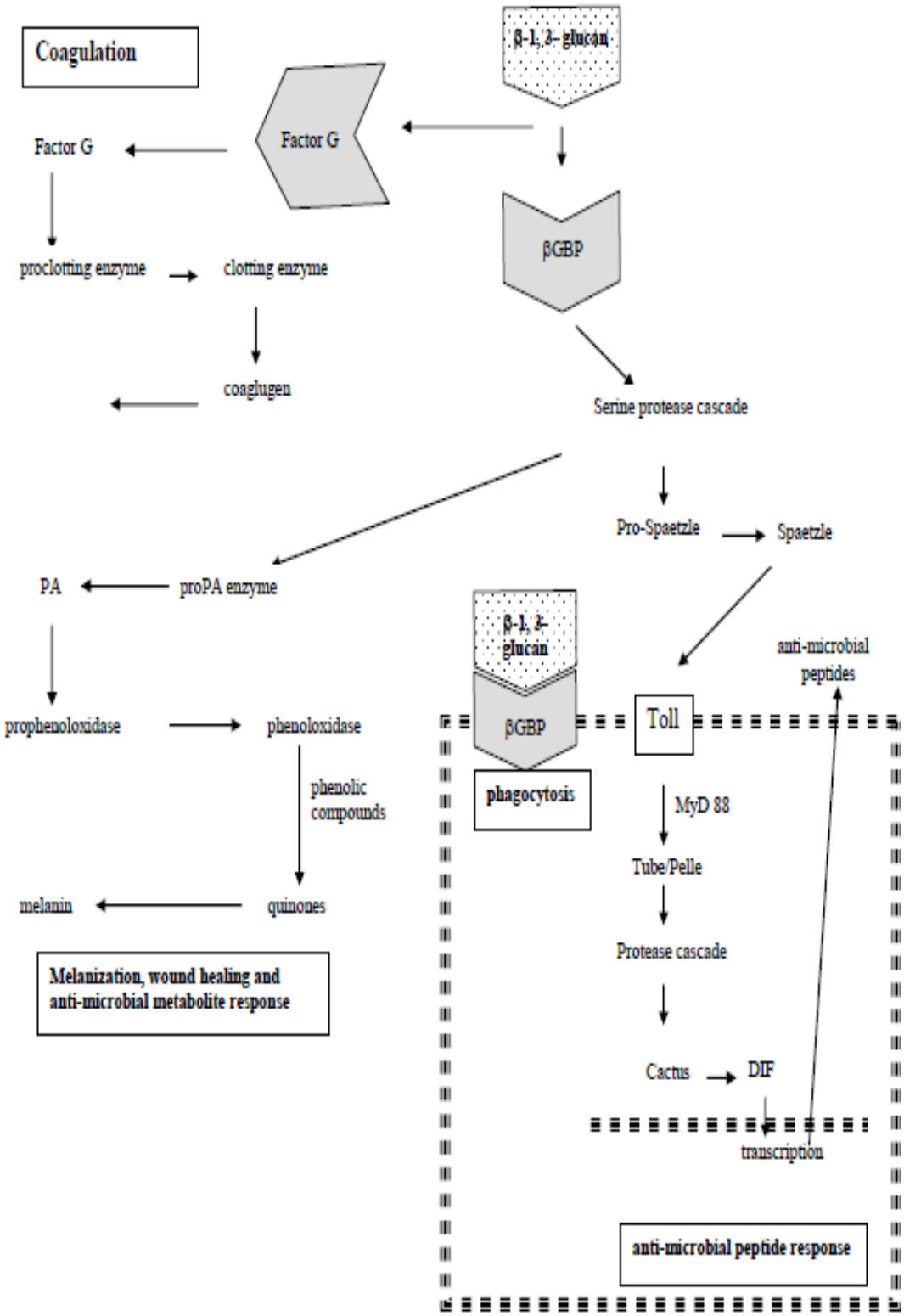

Figure 1.9: β-glucans induced responses and protease cascades in invertebrates. The β-glucans receptors are highlighted in grey, dotted line represents the fat body. Legend: PA: phenoloxidase activating enzyme; BGBP: beta-glucan binding protein. (Source: Soltanian *et al.*, 2009)

Their data suggest that specific immunological protection can be induced by a single exposure to a low dose of heat-killed bacteria, thus resembling the phenomenon of a vaccination. For decapods evidence has shown that the primed status might last between 3 and 72 h (Smith *et al*. 2003). This could indicate that the effect on the immune system is relatively short lived and the 'cost' can be high. Research on bumble bees showed that immune priming can extend for days and weeks, sometimes even across the different life stages (Schmid-Hempel, 2005).

The ecological cost of a heightened immune system is high, since energy will be lost for growth or reproduction. Therefore care should be taken with inappropriate priming, since it can eventually deplete the immune system of the host animal causing it to become exhausted and which can lead to mortality (Hauton & Smith, 2007; Rowley & Pope, 2012). Nevertheless not everyone is convinced that we can really talk about acquired immunity (Hauton & Smith, 2007). Arguments are being raised that a clear description of the underlying mechanisms together with reproducible evidence for multiple species is needed before any of these statements can be made, since most observations to date are very punctual and host-specific (Hauton & Smith, 2007).

1.5. Gnotobiotic model systems

1.5.1. History

Composed of two ancient Greek words, 'gnostos' or 'known' and 'bios' or 'life', gnotobiotic refers to an environment where only known strains of bacteria are present. Axenic conditions without any microorganisms in the system are also included in the bigger term gnotobiotic, but gnotobiotic does not equal axenic conditions. Gnotobiotic animals are very useful in studying host-microbe interactions since they provide researchers with a way to examine processes without interference or influence from unknown microbiota (Gordon & Pesti, 1971). The idea to develop gnotobiotic animals was put forward in the 19th century. In 1885, Louis Pasteur postulated that the animal host would not be able to survive without microbial associates. Based on the evolution theory 'survival of the fittest' he hypothesised that synergistic microbes are vital for the life of the host. Despite this theory, Reyniers and co-workers demonstrated in 1940 the first explicit proof for axenic animals using rats and

chickens, which settled the debate and provided future researchers with a new variety of possibilities (Gordon & Pesti, 1971).

1.5.2. Gnotobiotic models

It is doubtful that we will ever know everything there is to know about every living organism. Scientists realised this already a couple of centuries ago and started to focus on a couple of 'easy' animals and study them in great detail. Once they had answers to their questions focusing on one animal, extrapolation to related organisms could help solve of some of the important biological questions. Animal model systems were created to be used in research for better understanding human disease without the risk of harming a human being.
Some criteria are to be taken into account when selecting an animal model system. The animal in question should be of suitable size, easy to rear, not expensive and a short life cycle is a desirable quality. Nevertheless historical reasons also play a major role in the selection of model systems. Most of the best known model organisms are mammalian or vertebrate organisms (e.g. mouse, pig, rat, chicken & macaques); though the zebra fish has recently gained an extensive amount of attention as a model vertebrate system thanks to their resemblance to the mammal physiology. They are easy to manipulate genetically as well as to culture them in axenic/gnotobiotic conditions (Chu & Mazmanian, 2013).

There are a wide range of insect model systems available thanks to lesser ethical objections to the use of invertebrate animals. Due to their small size they can be easier grown in laboratory conditions and their relatively short generation time allows for much faster data generation over a short period of time. Some of the best known invertebrate animal model systems are the fruit fly (*Drosophila melanogaster*), the mosquito malaria model, *Hydra* spp. and the water flea (*Daphnia pulex*). Next to animal model systems there are also plant model systems (e.g. *Arabidopsis thaliana*) and bacterial (*Escherichia coli*) model system. The major constraints hampering the wide use of marine gnotobiotic organisms in research are the need for disinfection methods to produce germ free organisms and difficulties in assuring the complete germ free condition of a culture system (Marques *et al*., 2006). For fish, the best known animals used in gnotobiotic research are zebrafish, sea bass and the Atlantic cod model (Dierckens *et al*., 2009 Forberg *et al*., 2011). One thing that shouldn't be forgotten when working with gnotobiotic animals is the sterility checks and the problems related to the detection of contaminations.

1.5.3. Gnotobiotic brine shrimp (*Artemia franciscana)* as a model system

Artemia franciscana do not only serve as live feed for aquaculture purposes but they are also frequently being used as a gnotobiotic model system to study host-microbial interactions and immune responses. Several studies have already been done using them as a model organism for investigating the toxicological screening of chemical substances (Vanhaecke *et al*., 1981), the effect of chemotherapy agents against diseases occurring in penaeid shrimp, lobsters and other crustaceans (Overton & Bland, 1981; Criado-Fornelio *et al*., 1989; Verschuere *et al*., 1999 & 2000, Marques *et al*., 2004; Soltanian *et al*., 2007), pathogenic effect of bacteria under gnotobiotic conditions (Marques *et al*., 2004 & 2005) and the protective effect of heat shock proteins against vibriosis (Baruah, 2012).

Artemia franciscana are non-selective filter feeders that can ingest a maximum particle size of 25-30 µm for nauplii and 50 µm for adults (Figure 1.9). They can easily be cultured under gnotobiotic conditions in a laboratory environment and in high densities (Rojas-Garcia 2008; Marques *et al*., 2004; Soltanian *et al.*, 2007). There is a standardised protocol available to obtain sterile *Artemia* cysts and nauplii (Sorgeloos *et al*., 1986; Marques *et al*., 2004). All of these characteristics make them an ideal candidate for a gnotobiotic model system for marine crustaceans, provided that some other features are added to the research. For example, obtaining standardised lab strains rather than wild populations would provide multiple new research opportunities in the future.

A good model system also allows for genetic manipulation thanks to an annotated genome. This is the case for the zebra fish offering plenty of opportunities to investigate the molecular mechanisms underlying host-microbe interactions (Rawls *et al*., 2004). For *Artemia franciscana* there is a draft genome available (De Vos *et al*., 2013), although there is room for improvent it is usable. There are also studies available on RNAi in *Artemia franciscana* (Sung *et al*., 2007; King & MacRae, 2012; Copf *et al*., 2006). These two already allow some gene manipulation, yet the practice is not standardised yet. Once standardized, this will allow for the development of several gene manipulation techniques, for example together with the development of common garden experiments which are not always possible for the other available gnotobiotic model animals. Common garden experiments allow for testing different generations at the same time, via the haching of cysts of different generations.

1.5.4. onstraints of gnotobiotic culture

Axenic animals proved to have some major constraints. Underexposure to antigens leads to weaker cellular and humoral defence systems together with abnormalities in structure and function of various organs. Some of these organs were part of the immune system with no direct contact to the microbial associates, for example a lower cardiac output (Coates, 1975). This might implicate that the host is not self-sustainable and relies on microbial components for maintenance of their physiological state. When an axenic animal matures, they can show signs of weight loss, muscle atrophy, fatigue as well as an underdeveloped morphology otherwise known as the 'wasting syndrome'. These observations were done based on experiments on gnotobiotic mammals (mainly pigs), birds and zebra fishes (Coates, 1975; Rawls *et al*., 2004). Therefore one should be careful to extrapolate results from animals of axenic or gnotobiotic conditions to conventional animals since behaviour can be atypical and different parameters should be researched when using these animals, for example survival, growth, immunological responses and histological development (Marques *et al*., 2006; Gordon & Pesti, 1971; Coates, 1975).

Figure 1.9: 10-day old conventionally reared *Artemia franciscana* from Great Salt Lake

CHAPTER 2

THESIS OUTLINE & SCIENTIFIC AIMS

CHAPTER 2: Thesis outline & scientific aims

The overall objective of this thesis was to increase the scientific understanding of the immunological response of *Artemia franciscana* when being exposed to β-1.3/1.6-glucans. However, in order to investigate long-term effects on the host, we needed to prolong the gnotobiotic rearing period past the currently used nauplius instar II stage, using the axenic microalgae *Tetraselmis suecica* as a feed. Further, *Vibrio campbellii* LMG 21363, the pathogen routinely used in challenge tests at our laboratory proved not to induce sufficiently high mortality to perform a challenge test 2 days after hatching. A new and more virulent pathogen needed to be identified for the challenge tests.

The specific aims of this PhD study were:

1. To identify a pathogen that induces high levels of mortality in challenge tests during the whole life cycle of *Artemia franciscana*

2. To verify axenic microalgae as a feed for prolongation of the gnotobiotic *Artemia* system possibly up to adult stage. This was done in order to facilitate investigating the effect of the immunostimulant during the entire life span of the animal and to determine the effect of gnotobiotic growth on the gastrointestinal development of the animals

3. To establish the (priming) effect of β-1,3/1,6-glucans on survival and gene expression of immune-related genes in *Artemia franciscana*

In **Chapter 3,** *Vibrio* sp. H6 was identified as a virulent pathogen for brine shrimp. This strain was used in all further tests.

In **Chapter 4**, a comparative morphological study of the gastrointestinal tract of both gnotobiotically and conventionally reared *Artemia* nauplii of 2 and 4 days after hatching was performed, using fluorescence microscopy and transmission electron microscopy (TEM). This

was done to evaluate the axenic microalgae as a feed and the influence of the axenic conditions on the morphology of the brine shrimp reared in the gnotobiotic system.

In **Chapter 5**, a histological analysis was performed on conventionally reared animals (without challenge) to identify morphological alterations of the gastrointestinal tract of conventionally reared *Artemia franciscana* over a period of 8 days. This was done in order to explain observed changes in survival of conventional animals after challenge tests during the same time period of 8 days.

In **Chapter 6**, the effect of commercially available β-1.3/1.6-glucan particles from a specially-selected strain of *Saccharomyces cerevisiae* (MacroGard) on the survival of *Artemia* nauplii that were either or not challenged with *Vibrio* sp. H6 was investigated.

In **Chapter 7**, the impact of β-1.3/1.6-glucan particles on the expression of eight genes correlating with the innate immune response of *Artemia franciscana* was investigated using qPCR. The genes included the heat shock protein 70 gene (*hsp70*), prophenoloxidase (*proPO*), transglutaminase (*tgase*), masquarade like protein (*masq*), extracellular superoxide dismutase (*esod*), Peroxinectine (*pero*), lipopolysaccharide and β-1,3-glucan binding protein (*lgbp*), and Down syndrome cell adhesion molecule (*dscam*).

In **Chapter 8**, a general discussion, conclusion and some future perspectives are presented.

CHAPTER 3

CHARACTERISATION OF THE VIRULENCE OF *HARVEYI* CLADE VIBRIOS ISOLATED FROM A SHRIMP HATCHERY *IN VITRO* AND *IN VIVO,* IN A BRINE SHRIMP (*ARTEMIA FRANCISCANA*) MODEL SYSTEM

This chapter is based on:

Vanmaele S., Defoirdt T., Cleenwerck I., De Vos P., Bossier P. (2015) Characterization of the virulence of *Harveyi* clade vibrios isolated from a shrimp hatchery *in vitro* and *in vivo*, in a brine shrimp (*Artemia franciscana*) model system. *Aquaculture* **436**, 28-32

CHAPTER 3:

Characterisation of the virulence of *Harveyi* clade vibrios isolated from a shrimp hatchery *in vitro* and *in vivo*, in a brine shrimp (*Artemia franciscana*) model system

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ABSTRACT

Vibrios belonging to the *Harveyi* clade are important pathogens of a large number of marine animals in the aquaculture industry. In this study, six isolates (H1 to H6) were obtained from a shrimp hatchery in Rio Grande do Norte (Natal-Area, Brazil), which had been confronted with disease outbreaks in 2009. The aim was to characterise the virulence of these isolates, both *in vitro* (virulence factor production) and *in vivo* (virulence towards gnotobiotic brine shrimp, *Artemia franciscana*, larvae) and to compare these characteristics to those of the most virulent pathogen in the brine shrimp model described to date, *Vibrio campbellii* LMG 21363. Of all 6 isolates, H5 and H6 were found to be the most virulent ones and were therefore selected for further characterization. Isolate H5 exhibited a similar virulence as the control strain, while H6 exhibited a higher virulence, both in gnotobiotic and conventionally reared brine shrimp. Both H5 and H6 were motile and produced all of the lytic enzymes tested (hemolysin, caseinase, gelatinase, lipase and phospholipase). Although H6 was the most virulent isolate *in vivo*, this was not reflected in the highest production of all virulence factors tested. Finally, isolates H5 and H6 were identified to belong to the *Harveyi* clade of vibrios.

3.1. INTRODUCTION

Vibrios are Gram-negative bacteria that are ubiquitous in the marine environment and can be found free-living in the water column, as part of a biofilm, or in association with a host (Thompson *et al*., 2004). The *Harveyi* clade of vibrios is a subgroup containing major aquaculture pathogens (Ruwandeepika *et al*., 2012). Vibriosis causes severe economical losses in shrimp, finfish and mollusk cultivation worldwide (Austin & Zhang, 2006; FAO 2012; Defoirdt *et al*., 2007).

The pathogenicity mechanisms of vibrios belonging to the *Harveyi* clade remain largely unraveled; however some virulence indicators have been described. The infectious cycle of pathogenic bacteria includes entry of the pathogen, establishment and multiplication, thereby causing damage to host tissues and cells, and exit (Donnenberg, 2000). These different steps involve the expression of virulence factors -gene products that allow the pathogens to infect and damage the host (Defoirdt, 2013). The ability to colonise and adhere to host surfaces is an essential step in successful infection of a host, and flagellar motility is thought to enhance the initial interaction of a bacterium with a surface by enabling the cell to overcome negative electrostatic forces (McCarter, 2004). Hence, motility can give an indication of the potential of an isolate to colonize a host. A second important phenotype in colonization of the host is biofilm formation (Costerton *et al*., 1981). Biofilm formation in vibrios depends on several factors including flagella, pili and exopolysaccharide biosynthesis (Yildiz & Visick, 2009). Exopolysaccharides form a loose slime outside the cell that forms an intercellular matrix in biofilms, which enhances the growth and survival of microorganisms by providing access to nutrients and protection from detergents or antimicrobials, predators and drying (Donlan & Costerton, 2002). A third group of virulence factors includes lytic enzymes, which are produced by many pathogenic bacteria and often play a central role in pathogenesis (Finlay & Falkow, 1997). These enzymes cause damage to host tissues, thereby allowing the pathogen to obtain nutrients and to spread through tissues. Lytic enzymes produced by pathogenic vibrios include hemolysins, proteases (including caseinase and gelatinase), and (phospho) lipases (Defoirdt, 2013).

In 2009, 6 *Vibrio* strains (H1-6) were isolated from a shrimp hatchery in Rio Grande do Norte (Natal-Area, Brazil), where high mortalities were faced at that moment. In this study,

the aim was to characterise the virulence of these isolates, by determining the production of some important virulence factors *in vitro*, and by determining their virulence towards gnotobiotic and conventionally reared brine shrimp (*Artemia franciscana*), a well-established model organism with respect to infections caused by *Vibrio* species in crustaceans (Soto-Rodriguez *et al*., 2003; Austin *et al*., 2005; Defoirdt *et al*., 2006).

3.2. MATERIALS & METHODS

3.2.1. Bacterial strains & growth conditions

The isolates H1-6 were obtained from a hatchery in Rio Grande do Norte in 2009 and were originally isolated from pacific white shrimp *Penaeus vannamei* (Natal-Area, Brazil). The *Vibrio campbellii* strain LMG 21363 and the six isolates were stored in 30% glycerol at -80° C. All bacterial strains were grown in marine broth (DifcoTM) at 28°C with constant agitation. Bacterial densities were determined spectrophotometrically at 550 nm. The bacterial densities were calculated according to the McFarland standard (BioMerieux, Marcy L'Etoile, France), assuming that an OD550=1.000 corresponds to 1.2×10^9 cells/ml.

3.2.2. Identification of the bacterial strains H5 and H6

A partial 16S rRNA gene sequence was amplified for strains H5 (1135 bp) and H6 (988 bp) as described previously (Castro *et al*., 2013). The conserved primers pA (5' AGAGTTTGATCCTGGCTCAG 3') and pH (5' AAGGAGGTGATCCAGCCGCA 3') were used to obtain the amplification products, while the primers *Gamma, Gamma and BKL1 (Coenye *et al*., 1999; Cleenwerck *et al*., 2007) were used to obtain the sequences. The partial sequences of both strains were compared with nearly complete 16S rRNA gene sequences of the type strains of the established *Vibrio* species retrieved from the EMBL database using the BioNumerics 5.1 software (Applied Maths, Belgium). A phylogenetic tree was constructed with the BioNumerics 5.1 software (Applied Maths) using the Neighbour-Joining method (Saitou & Nei, 1987).

3.2.3. Gnotobiotic culture of brine shrimp & challenge tests

All experiments were performed with *Artemia franciscana* cysts, originating from Great Salt Lake, Utah, USA (INVE Aquaculture NV, Belgium). Bacteria-free cysts and larvae were

obtained via decapsulation according to the procedure described by Sorgeloos *et al* (1986). *Artemia* cysts were hydrated in 9 mL of distilled water for 1 h. Sterile cysts and nauplii were obtained via decapsulation using 0.33 mL NaOH (32%) and 5 mL NaOCl (50%). During the reaction, 0.22 µm filtered aeration was provided. The decapsulation was stopped after about 2 min by adding 5 mL $Na_2S_2O_3$ (10 g l^{-1}). The aeration was then stopped and the decapsulated cysts were washed with filtered (0.22 µm) and autoclaved artificial seawater containing 35 g l-1 of Instant Ocean synthetic sea salt (Aquarium Systems, Sarrebourg, France. All procedures were performed under a laminar flow hood to maintain axenic conditions. All equipment was previously sterilised and autoclaved at 120°C for 20 min. Decapsulated cysts were washed several times over a 100 µm sieve with sterile Instant Ocean (35 g l^{-1}) and carefully capped. The tubes were placed on a rotor at four cycles per minute and constantly exposed to an incandescent light at 28°C for 18 – 22 h. Groups of 20 larvae (instar II stage nauplii) were collected and transferred to sterile glass tubes with Filtered Autoclaved seawater (35 g l^{-1}).

Isolates of the bacterial strains were aseptically inoculated in 30 ml Difco™ marine broth 2216 and incubated overnight at 25°-28°C with constant agitation. 150 µl was subsequently transferred and grown to stationery phase in 30 ml marine broth 6 h before challenge. The bacterial densities were determined spectrophotometrically at an optical density of 550 nm. The challenge test was performed according to Marques *et al*. (2006), no feed was added. The bacterial suspension was added to reach a final density of 10^7 cells ml^{-1}. Each treatment consisted out of 4 replicates. The survival of the brine shrimp larvae was determined after 48h according to Amend (1981). The relative percentage of survival (RPS) was calculated as follows:

RPS (%) = (% of surviving challenged larvae)/ (% of surviving unchallenged larvae) x 100

Axenity of non-challenged brine shrimp cultures was verified by transferring 100 µl of culture medium to petri dishes containing Marine agar 2216 (n=3). Plates were stored in an incubator for five days at 28°C. Only experiments of which the non-challenged cultures were confirmed to be axenic were considered for analysis.

3.2.4. Conventional culturing of brine shrimp & challenge tests

The same *Artemia franciscana* cysts as the gnotobiotic setup were used in the conventional experiments. Cysts were hatched in salt water with strong aeration and constant

illumination at 28°C during 18 – 22 h. Groups of 20 larvae (instar II stage nauplii) were collected and transferred to glass tubes with salt water for the duration of the challenge test. The conventional challenge test was performed in the same way as previously described for the gnotobiotic challenge. Each treatment consisted out of 4 replicates.

3.2.5. Virulence factor assays

Swimming motility and the production of the virulence factors caseinase, gelatinase, lipase, hemolysin and phospholipase were determined as described by Yang & Defoirdt (2014) and Natrah *et al*. (2011). The motility assay was done using soft agar plates (2% agar) with LB medium (10g NaCl). The diameter of the swimming zone was measured after 24 h incubation at 28°C. For the lipase and phospholipase assays, marine agar plates were supplemented with 1% Tween 80 (Sigma-Aldrich) or 1% egg yolk emulsion (Sigma-Aldrich), respectively. The development of opalescent zones around the colonies was observed and the diameter of the zones was measured after 2-4 days of incubation at 28°C. The caseinase assay plates were prepared by mixing double strength MA with a 4% skim milk powder suspension (Oxoid, Basingstoke, Hampshire, UK), sterilised separately at 121°C for 5 min. Clearing zones surrounding the bacterial colonies were measured after 2 days of incubation at 28°C. Gelatinase assay plates were prepared by mixing 0.5% gelatin (Sigma-Aldrich) into MA. After incubation for 7 days, saturated ammonium sulfate (80%) in distilled water was poured over the plates and after 2 min, the diameters of the clearing zones around the colonies were measured. Hemolytic assay plates were prepared by supplementing MA with 5% defibrinated sheep blood (Oxoid) and clearing zones were measured after 2 days of incubation at 28°C. All treatments were performed in triplicate.

3.2.6. Antibiotic susceptibility testing

Sensitivity to four antibiotics was determined using a disk diffusion assay. This assay was done using four antibiotic susceptibility test discs: ampicillin (10 µg), kanamycin (30 µg), tetracycline (30µg) and chloramphenicol (30µg). Each of the four discs was placed on a marine agar plate previously covered with one of the four bacterial isolates. The growth inhibition zone was measured after 24 h incubation at 28°C. All treatments were performed in triplicate.

3.2.7. Statistical analysis

Data analysis was done by independent samples t-tests or ANOVA with a Tukey's post hoc analysis. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 21.0 using a significance level of 5%.

3.3. RESULTS

3.3.1. Virulence of the isolates towards brine shrimp larvae

In a first experiment, we investigated the virulence of the 6 isolates towards gnotobiotic brine shrimp larvae. *V. campbellii* LMG 21363 was used as a control strain. All isolates were found to cause significant mortality (Figure 3.1). Isolate H3 was significantly less virulent than the other strains. Strains H5 and H6 were significantly more virulent than the other isolates, with H6 tending to be more virulent than the control strain LMG 21363 (although the difference was not significant in this analysis). We decided to focus on isolates H5 and H6 in further experiments because they were the most virulent.

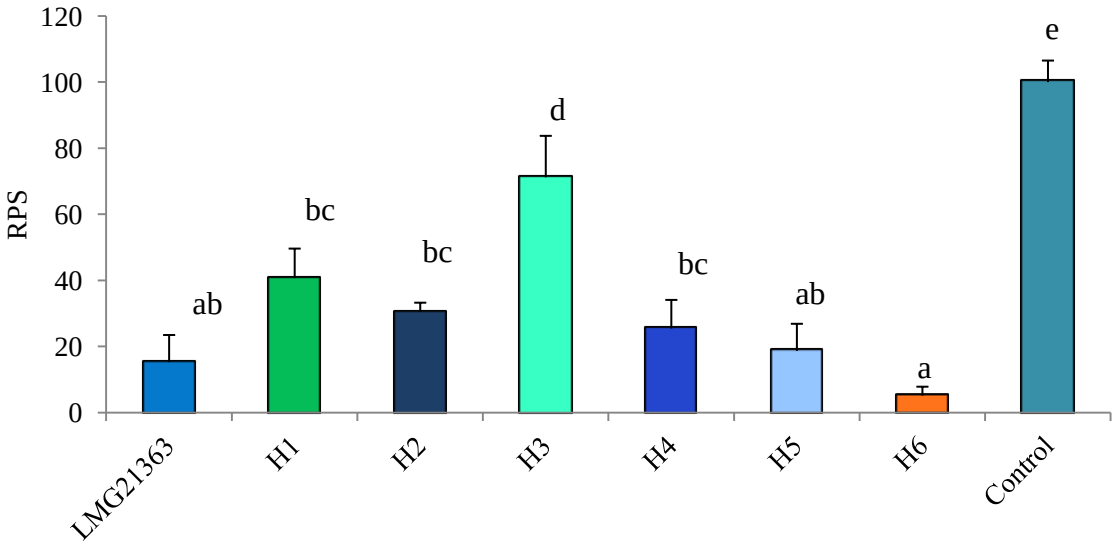

Figure 3.1: Relative percentage of survival (RPS) of gnotobiotic brine shrimp larvae 48 h after challenge with the isolates H1-6 and the known pathogen *Vibrio campbellii* LMG 21363. Error bars represent the standard deviation of 4 replicates. Independent samples t-tests were performed. Bars with different letters are significantly different ($P < 0.05$). "Control" refers to animals that were not challenged, but otherwise treated in the same way as in the other treatments.

In a second experiment, we further investigated the virulence of isolates H5 and H6 towards conventionally reared brine shrimp larvae. *V. campbellii* LMG 21363 was again used as control strain. H6 showed the highest mortality and statistical analysis showed that mortality caused by H6 was significantly different from the control strain *V. campbellii* LMG21363 and isolate H5 (Figure 3.2). These results were confirmed multiple times under both conventional and gnotobiotic conditions (data not shown).

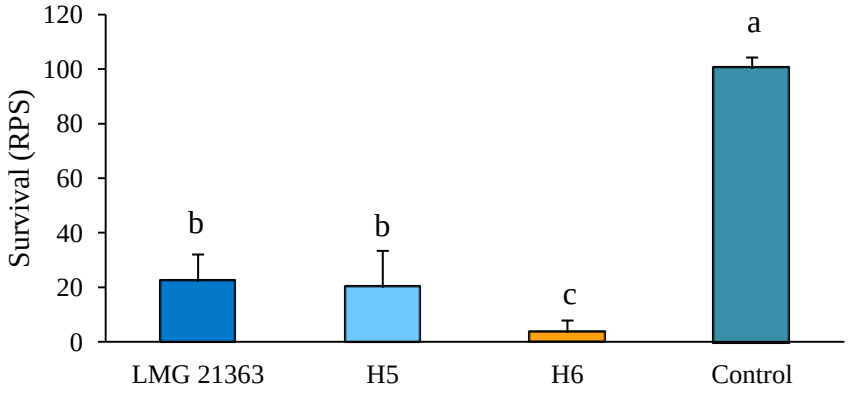

Figure 3.2: Relative percentage of survival (RPS) of conventionally reared brine shrimp larvae 48 h after challenge with the isolates H5 and H6 and the known pathogen *Vibrio campbellii* LMG 21363. Error bars represent the standard deviation of 4 replicates. One-way ANOVA was performed. Bars with different letters are significantly different ($P < 0.05$). "Control" refers to animals that were not challenged, but otherwise treated in the same way as in the other treatments.

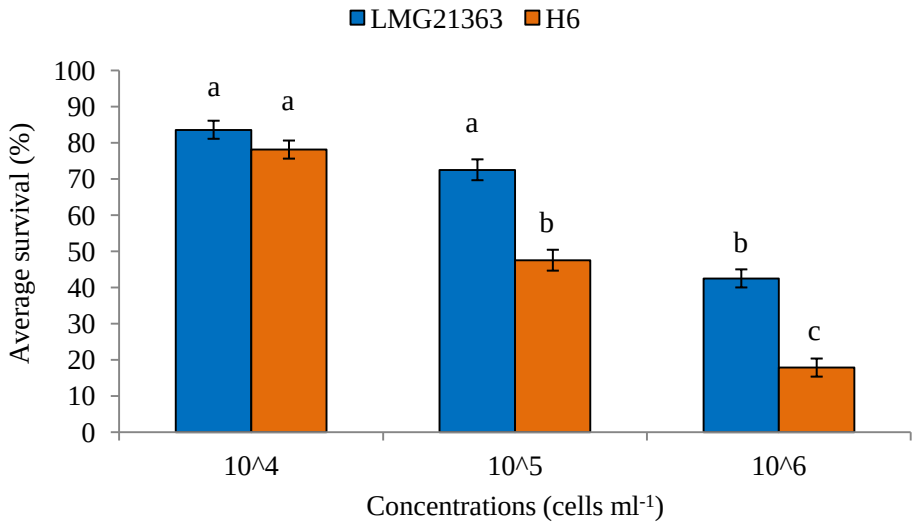

Figure 3.3: Average survival percentage of gnotobiotically reared brine shrimp larvae 48 h after challenge with *Vibrio campbellii* LMG 21363 and *Vibrio* sp. H6. Error bars represent the standard deviation of 4 replicates. Bars with different letters are significantly different (Independent sample t-tests; $P < 0.05$).

Different concentrations of the pathogen were added to the gnotobiotically reared brine shrimp in order to test the effect of inoculum concentration in the absence of feed (Figure 3.3). At a concentration of 10^4 cells ml^{-1} no significant difference was observed between both pathogens, overall survival was high (around 80%). A significant difference in survival of larvae challenged to both strains was observed at 10^5 cells ml^{-1}. At 10^6 cells ml^{-1} survival rates of around 15-20% were observed for larvae challenged with isolate H6 and therefore it was decided to use this concentration in further tests.

3.3.2. Growth rate and antibiotic susceptibility testing

Growth curve analysis showed that both H5 and H6 grow slightly slower than LMG 21363 (Figure 3.4). The antibiotic susceptibility test showed that strain LMG 21363 is resistant against ampicillin and tetracycline (Table 3.1). H5 was resistant against ampicillin only, while H6 was sensitive to all four antibiotics tested.

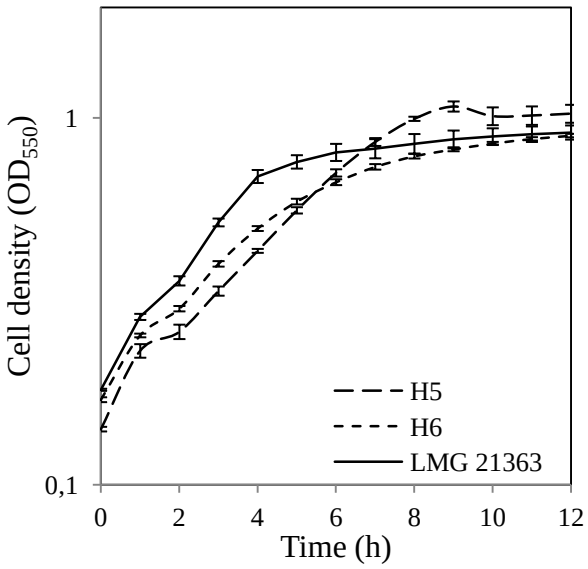

Figure 3.4: Growth curves of *Vibrio campbellii* LMG 21363, and isolates H5 and H6 in Marine broth. Error bars represent the standard deviation of 5 replicates.

Table 3.1: Inhibition zones of isolates H5 and H6, and control strain LMG 21363 for 4 antibiotics (Average ± standard deviation of three replicates).

Strain	**Inhibition zone (mm)**			
	Ampicillin	**Kanamycin**	**Tetracyclin**	**Chloramphenicol**
LMG 21363	0 ± 0	8 ± 1	0 ± 0	18 ± 1
H5	0 ± 0	11 ± 1	10 ± 2	23 ± 1
H6	13 ± 1	11 ± 1	11 ± 0	21 ± 1

3.3.3. Virulence factor tests

Several phenotypes have been linked with the virulence of vibrios (Liu *et al*., 2013; Ruwandeepika *et al*., 2012). In this study, we investigated swimming motility and lytic enzyme production (hemolysin, the proteases caseinase and gelatinase, lipase, and phospholipase). The motility test (Table 3.2) showed that H5 and H6 had a similar swimming motility, which was roughly half of the motility observed for the control strain LMG 21363. A significantly stronger hemolytic activity was observed for H5 when compared to H6 (Table 3.3) and for both H5 and H6 when compared to LMG 21363. The caseinase activity of isolate H6 was slightly lower when compared to LMG 21363 and H5. No significant difference was observed between H5 and LMG 21363. Lipase activities of both H5 and H6 were significantly higher than that of LMG 21363. For gelatinase, the strongest production was observed for H6 but it was in the same order as observed for LMG 21363. The production of gelatinase was significantly lower for H5. Finally, no significant differences were observed in phospholipase activity between the three strains.

Table 3.2: Swimming motility zone of isolates H5 and H6, and control strain LMG 21363 on soft agar after 24 h incubation (Average ± standard deviation of three replicates).

Strain	**Swimming zone (mm)**
LMG 21363	67 ± 3
H5	39 ± 6
H6	35 ± 3

Table 3.3: Virulence factor activity of isolates H5 and H6, and control strain LMG 21363. (Average ± standard deviation of three replicates). For each virulence factor, different superscript letters indicate significant differences (independent samples t-test; $P < 0.05$).

Strain	Activity Zone (mm)	Colony diameter (mm)	Ratio
		Haemolysin	
LMG 21363	20 ± 0	10 ± 0	2.0 ± 0.0^{A}
H5	37 ± 4	12 ± 1	3.0 ± 0.3^{B}
H6	28 ± 2	11 ± 1	2.5 ± 0.1^{B}
		Caseinase	
LMG 21363	22 ± 1	15 ± 1	1.4 ± 0.0^{A}
H5	21 ± 1	15 ± 1	1.4 ± 0.0^{A}
H6	21 ± 1	16 ± 1	1.3 ± 0.0^{B}
		Lipase	
LMG 21363	23 ± 1	15 ± 1	1.5 ± 0.1^{A}
H5	32 ± 1	11 ± 0	2.9 ± 0.1^{B}
H6	32 ± 1	11 ± 1	3.0 ± 0.3^{B}
		Phospholipase	
LMG 21363	24 ± 1	11 ± 1	2.3 ± 0.1
H5	24 ± 3	10 ± 1	2.4 ± 0.2
H6	26 ± 1	11 ± 0	2.4 ± 0.1
		Gelatinase	
LMG 21363	93 ± 6	5 ± 0	19.0 ± 1.5
H5	100 ± 0	6 ± 0	17.8 ± 0.2
H6	113 ± 6	6 ± 0	20.4 ± 0.9

3.3.4. Identification of the bacterial strains H5 and H6

A preliminary identification has been done for both H5 and H6 (Figure 3.5). Species of the *Vibrio harveyi* group (Yoshizawa *et al*., 2009) were found as the closest relatives, with strain H5 exhibiting the highest pairwise sequence similarity with *Vibrio owensii* (99.9%), *Vibrio rotiferianus* (99.8%) and *Vibrio jasicida* (99.5%), and strain H6 with *Vibrio natriegens* (99.1%).

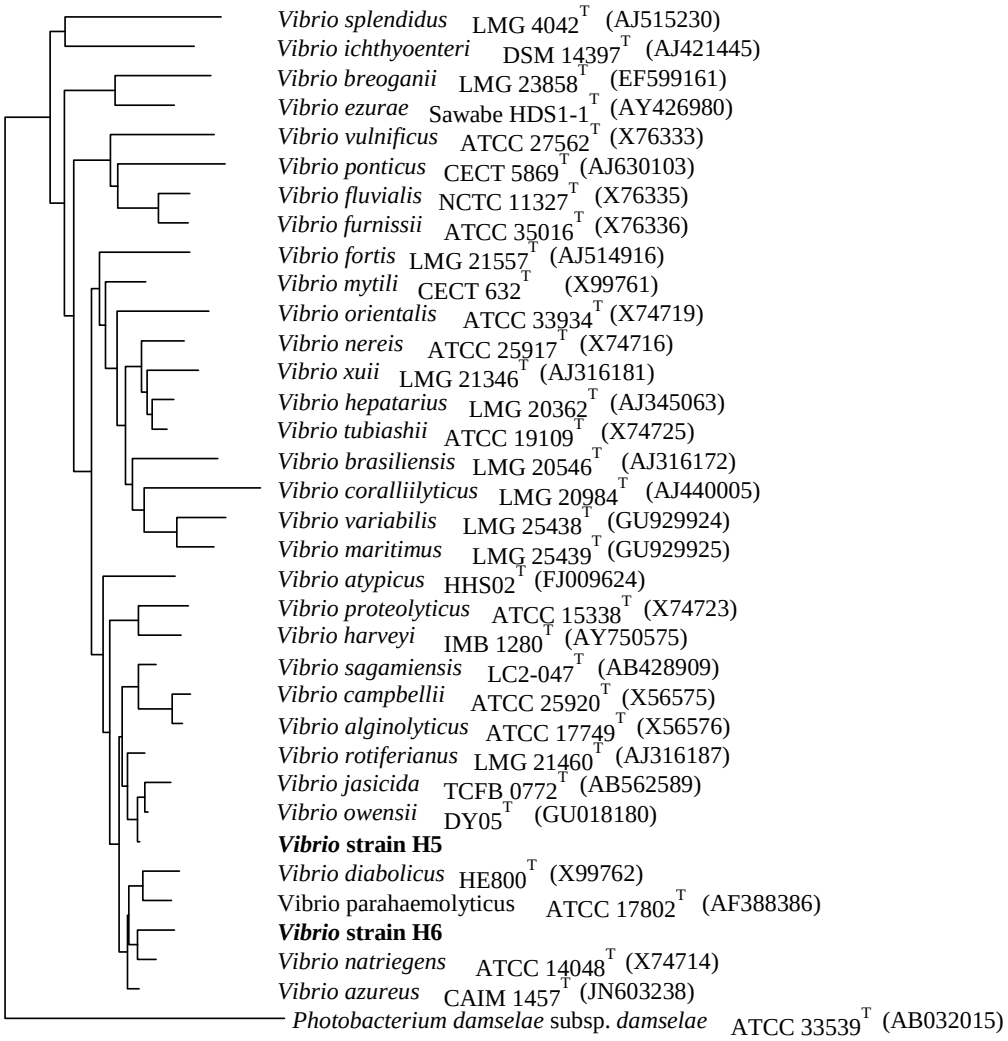

Figure 3.5: Phylogenetic tree constructed using the Neighbour-joining method and based mainly on nearly complete 16S rRNA gene sequences. The type strain of *Photobacterium damselae* subsp. *damselae* ATCC 33539T is used as outgroup. Bar, 1 nt substitution per 100 nt.

3.4. DISCUSSION

Bacterial diseases cause massive mortalities in fish, shellfish and mollusc cultures worldwide (Liu *et al*., 2013). One group of pathogens responsible for high economical losses especially in shrimp cultures are vibrios belonging to the Harveyi clade (Austin & Zhang 2006; Ruwandeepika *et al*., 2012). However, not all strains belonging to the *Harveyi* clade are

pathogenic, studies showed that pathogenicity is not associated with specific species, but is rather a strain characteristic (Austin & Zhang 2006; Ruwandeepika *et al*. 2010).

In this study we described 6 *Vibrio* strains isolated from a shrimp hatchery. In a first experiment, we investigated the virulence of the 6 isolates towards gnotobiotic brine shrimp larvae and compared this with the virulence of *V. campbellii* LMG 21363, the most virulent isolate in the brine shrimp model reported to date. Based on this, the two most virulent isolates, H5 and H6, were selected for further experiments. One of the isolates, *Vibrio* sp. H6, induced significantly higher mortality in the brine shrimp during the challenge tests and is therefore as far as we know the most virulent strain in the brine shrimp model described thus far.

Although vibrios belonging to the Harveyi clade are widely know for the diseases they cause, not much is know yet on the pathogenicity mechanisms and mode of action. Several virulence factors have been described to be associated with virulence of bacteria belonging to the *Harveyi* clade (Liu *et al*., 2013; Packiavathy *et al.,* 2012). Lytic enzymes (haemolysin, lipases, etc.) are known to cause severe damage to host tissues, and this allows the bacteria to acquire nutrients from the host and spread through the body. Haemolytic activity for both isolates was significantly higher when compared to *Vibrio campbellii* LMG21363; and while H6 proved to be more virulent, H5 had the highest haemolytic production. Significantly higher levels of lipase production were also observed for both isolates when compared to *Vibrio campbellii* LMG21363. High levels of lipase and phospholisase have previously been reported as being correlated with the capacity of the bacteria to induce disease in marine vertebrates (Mendez *et al.,* 2012). Caseinase production for H6 was lower than the level produced by *Vibrio campbellii* LMG21363.

Increasing disease outbreaks attributed to bacteria belonging to the *Vibrio* genus and the development of antibiotic resistance of several strains has turned the interest of researchers towards alternative stragies for solutions against vibriosis both in prevention and treatment (Defoirdt *et al*. 2011; Ruwandeepika *et al*., 2012). Development of these new strategies can highly benefit from knowledge of the production of virulence factors associated with the pathogenicity of the strains, e.g. inhibition of their gene expression.

3.5. CONCLUSION

In conclusion, both isolates were positive for all virulence factors tested. However, although isolate H6 was the most virulent strain *in vivo*, it did not show the highest activity for all virulence factors or in motility activity. Further research is recommended to determine the cause for this high virulence compared to *Vibrio campbellii* LMG 21363 and to further indentify the isolate H6.

CHAPTER 4

A COMPARATIVE STUDY OF THE GASTROINTESTINAL TRACT OF GNOTOBIOTIC *ARTEMIA FRANCISCANA* VERSUS THEIR CONVENTIONAL COUNTERPART

In collaboration with Prof. dr. ir Peter Bossier and Prof. dr. Wim Van Den Broeck

CHAPTER 4:

A comparative study of the gastrointestinal tract of gnotobiotic *Artemia franciscana* versus their conventional counterpart

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ABSTRACT

Gnotobiotic animals provide a way to examine host-microbe interactions without interference or influence from complex and unknown microbial communities present in the natural environment. For marine organisms it is very difficult to develop these gnotobiotic conditions, especially to obtain axenic animals to work with. Thanks to its unique properties *Artemia franciscana* is perfectly suited as a marine invertebrate model animal. Nevertheless axenic conditions prove to have major constraints as for example abnormalities in structure and function of various organs are just some of the limitations that can occur. The aim of this study was to identify if morphological impediments in gut development, previously identified in other animals, pose a constraint for culturing older gnotobiotic *Artemia*. Gnotobiotic *Artemia* nauplii fed with axenic microalgae were sampled at day 2 and day 4 and were compared to their conventional counterparts and the changes in histology and cellular morphology were determined. Light and electron microscopic analyses were performed, together with measurements of the total length of the animals and cell heights of the gastrointestinal wall. Results showed that differences between gnotobiotic and conventional animals were minor at day 2. On the contrary results at day 4 showed something very different. This can be seen in the total length of the animals, being only half for axenic animals ones *vs*. conventional animals, as well as in the deterioration of the epithelial cells and the microvilli brush border of the alimentary tract. In conclusion, the gut integrity was preserved in nauplii fed with axenic microalgae until day 2 of their development, however no such conclusion can be made for the gnotobiotic treatment as animals grow older.

4.1. INTRODUCTION

Gnotobiotic animal model systems were originally developed to be used in research as surrogate for human experiments. The animal should be of suitable size, easy to rear, not expensive and have a short life cycle. Most of the best known model organisms are mammalian or vertebrate organisms (e.g. mouse, pig, rat, chicken & macaques); though the zebra fish has recently gained an extensive amount of attention as a model vertebrate system thanks to their similarity in physiology with mammals (Chu & Mazmanian, 2013). There are also a wide range of insect model systems available thanks to the absence of ethical objections. One of the best known invertebrate animal model systems is the fruit fly. There are a wide range of insect model systems available thanks to lesser ethical objections to the use of invertebrate animals. One of the best known invertebrate animal model systems is the fruit fly.

Gnotobiotic conditions refer to an environment where only known strains of bacteria or other microorganisms are present. It allows researchers to investigate the desired host-microbe interactions without interference from complex and unknown microbial communities that are present in the natural environment. However, for marine organisms it is difficult to obtain gnotobiotic organisms, because the disinfection methods are not always safe for the eggs or larvae to produce germ-free organisms (Marques *et al*., 2006). Problems occur however as gnotobiotic animals grow older, they can show signs of weight loss, muscle atrophy and fatigue, which is known as the 'wasting syndrome'. Underexposure to antigens leads to weaker cellular and humoral defence systems together with abnormalities in structure and function of various organs, especially in the gastrointestinal tract (Thompson & Trexler, 1971). This implicates that the host is not self-sustainable and depends on microbial activity for the maintenance of an optimal physiological state. This has been observed in all gnotobiotic model systems that have been used up to now (Coates, 1975). That is why it is important to investigate whether the gnotobiotic state and the correlated impediments in development pose a constraint to use older animals in host-microbe experiments (Marques *et al*., 2006).

The brine shrimp *Artemia franciscana* is a small branchiopod crustacean that can be found in natural saline lakes and solar salt works (Clegg & Trotman, 2002). *Artemia* is a non-

selective particle filter feeder that can ingest a maximum particle size of 25-30 µm for nauplii and 50 µm for adults. They are able to live on a variety of feeds, for example microalgae (e.g. *Tetraselmis suecica*) or baker's yeast (*Saccharomyces cerevisiae*) (Marques *et al*., 2004; Marques *et al*., 2006). The morphology of gnotobiotic and conventionally reared *Artemia* nauplii has previously been described. The gut morphology of conventional *Artemia* nauplii was studied in detail using transmission electron microscopy a few decades ago by Hootman and Conte (1974). A second study was performed in 1991 by Godelieve Criel. She described the morphology of conventional *Artemia* using scanning electron microscopy and light microscopy. Gunasekara (2011) described the histology, three-dimensional architecture and cellular morphology of the gut of gnotobiotic *Artemia* nauplii.

The gastrointestinal tract of gnotobiotic *Artemia* nauplii looks like a hooked, tubular structure freely suspended in the haemolymph. It is composed out of three parts: the foregut, midgut and hindgut. The mid- and hindgut are aligned, where the foregut is a hooked tube with a shorter part perpendicular to the midgut. The length and the diameter of the fore- and hindgut are smaller compared to those of the midgut. There are also two globular protrusions present at the anterior part of the midgut, known as gastric or hepatopancreatic caeca (Gunasekara *et al*., 2011). A single layer of epithelial cells borders the alimentary tract. The enterocytes of the foregut and hindgut are cuboidal, lined by a thin cuticle and have mainly mechanical functions. Midgut enterocytes are cuboidal to columnar and possess an apical microvilli brush border for absorption, storage and secretion purposes (Gunasekara *et al*., 2011).

In the study of Gunasekara (2011), *Artemia* nauplii were fed with a selected strain of *Aeromonas hydrophila* LVS3 in combination with different yeast cells. However, the current zootechnical approach of rearing gnotobiotic *Artemia* and the feeding regime does not allow growing adult *Artemia* under gnotobiotic conditions. At present it is not known under which gnotobiotic conditions adult *Artemia* can routinely be obtained. It has been hypothesised that the LVS3-*Aeromonas* particles being used in the culturing system, are an inadequate feed. It was assumed to be one of the reasons why the culture could not be prolonged.

The aim of this study was to evaluate the axenic microalgae *Tetraselmis suecica,* because of its high nutritional properties, as a feed for obtaining adult gnotobiotic *Artemia* (Serdar *et*

al., 2007). A comparative morphological study was performed for the histology and cellular morphology of the gut of gnotobiotic and conventional *Artemia* nauplii, during the early stages of their life cycle, to see whether or not severe morphological impediments are in place during their development. Nauplii were sampled on day 2 and day 4 after hatching. Light and electron microscopic analyses were performed, together with measurements of the total length of the animals and the cell height of the gastrointestinal wall. To our knowledge it is the first time that a comparison of the development of the gastrointestinal tract of both gnotobiotic and conventional *Artemia* nauplii under these conditions has been described.

4.2. MATERIALS & METHODS

4.2.1. Culturing & harvesting of the axenic microalgae

The axenic strain of *Tetraselmis suecica* 66/4 was obtained from the Culture Collection of Algae and Protozoa (CCAP, Dunstaffnage Marine Laboratory, Scotland). The algae were grown in WALNE medium (Walne, 1967) using sterile 500 ml or 1000 ml Schott bottles, starting with 10% inocula, and provided with 0.22 μm filtered aeration. Non-axenic cultures of the same algae strain were used for the conventional experiments. All parameters for algal cultures were kept at a constant pH of 7, continuous light of 100 μM photons $m^{-2}s^{-1}$, a temperature of 24°C and 30 g l^{-1} salinity. The density of the cultures was measured using a Bürker hemocytometer.

4.2.2. Gnotobiotic culture of brine shrimp

All experiments were performed with *Artemia franciscana* cysts, originating from Great Salt Lake, Utah, USA (INVE Aquaculture NV, Belgium). Bacteria-free cysts and larvae were obtained through decapsulation according to the procedure described by Sorgeloos *et al.* (1986). *Artemia* cysts were hydrated in 9 ml of distilled water for 1 h. Sterile cysts and nauplii were obtained via decapsulation using 0.33 mL NaOH (32%) and 5 ml NaOCl (50%). During the reaction, 0.22 μm filtered aeration was provided. The decapsulation was stopped after about 2 min by adding 5 ml $Na_2S_2O_3$ (10 g l^{-1}). The aeration was then stopped and the decapsulated cysts were washed with filtered (0.22 μm) and autoclaved artificial seawater containing 35 g l^{-1} of Instant Ocean synthetic sea salt (Aquarium Systems, Sarrebourg,

France). All procedures were performed under a laminar flow hood to maintain axenic conditions. All equipment was previously sterilised and autoclaved at 120°C for 20 min. Decapsulated cysts were washed several times over a 100 µm sieve with sterile Instant Ocean (35 g l^{-1}) and resuspended in a 50 ml glass tube containing 30 ml filtered and autoclaved artificial seawater. The glass tubes were placed on a rotor at four cycles per minute and constantly exposed to an incandescent light at 28°C for 18 – 22 h, allowing the emerged nauplii to reach stage II in which they are able to ingest bacteria. After 22h, groups of 20 larvae (instar II stage nauplii) were collected and transferred to sterile glass tubes with filtered autoclaved sea water (35 g l^{-1}). For morphological analysis and growth measurements 15 animals were collected at day 2 and day 4. There was not sufficient survival of the axenic animals after day 4 to further continue with the sampling.

4.2.3. Axenity testing of the brine shrimp and microalgae cultures

Axenity of the brine shrimp cultures was verified by transferring 100 µl of culture medium to petri dishes containing marine agar 2216 (n=3). Plates were stored in an incubator for five days at 28°C. If treatments were found to be contaminated, results were not considered. Axenity testing of the microalgae was done by plating the supernatant on marine agar and by flow cytometry. Samples were also routinely checked microscopically for contamination at 1000x magnification with oil immersion, immediately before harvesting.

An Accuri C6 flow cytometer (BD Biosciences) was used to verify axenity of the microalgae (Figure 4.1). The flow cytometer was equipped with an autoloader (Van Nevel *et al*., 2013). The blue laser of the flow cytometer (488nm) detects chlorophyll b, while the red laser (640nm) is for both chlorophyll a and b. The FL3 detector catches the light from the blue laser (light >640nm) and the FL4 detects the light coming from the red laser (665-685nm). For detecting bacteria, samples were stained using different compounds. They were stained using 'Live Death' staining, which is a combination of SYBR Green I and propidium iodide (SG, 10,000× diluted from stock; PI, 3 µM final concentration; Invitrogen) or SYBR Green I (SG, 10,000× diluted from stock; Invitrogen). Both the Live Dead staining and SYBR Green I are fluorescent and bind to the DNA of bacteria, while the propidium iodide can only enter damaged cells. The graph on the left in Figure 4.1 shows the presence of the algae *Tetraselmis suecica* 66/4 in the sample, occurring high on both the FL4 and the FSC axis (staining with Live Dead). The graph in the middle shows absence of bacteria in the axenic sample (staining

with Live Dead). Finally, the graph on the right shows presence of bacteria in the non-axenic sample of the same algae strain. For the last graph (right), the samples were stained using SYBR Green I (SG, 10,000× diluted from stock; Invitrogen). Results showed that the microalgae cultures were kept axenic during the experiment.

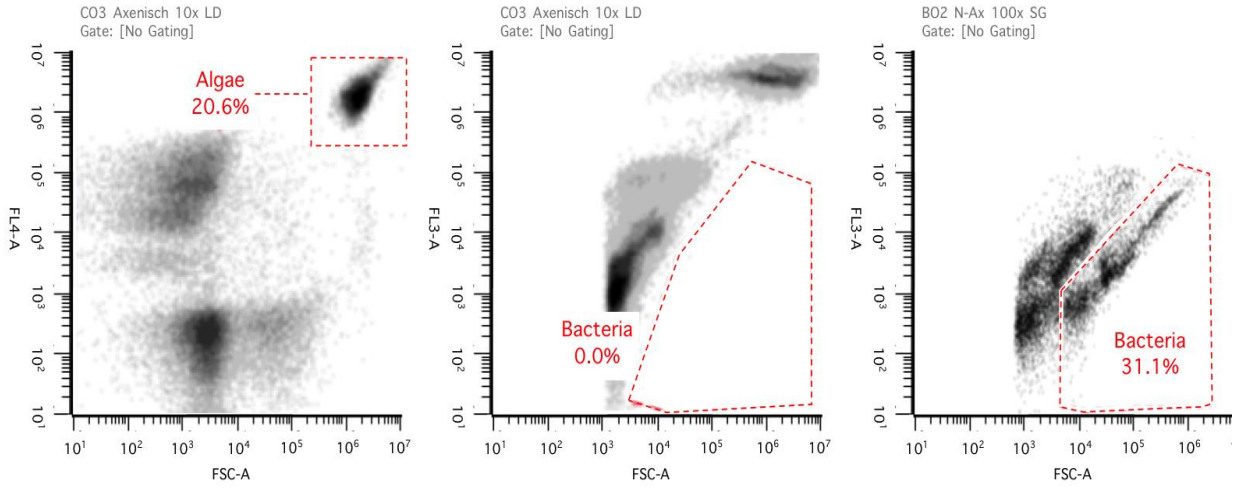

Figure 4.1: Results of the flow cytometry analysis. FSC: forward scatter; FL4: red laser (640 nm); FL3: blue laser (488nm), LD: Live Dead staining, SG: SYBR green.

4.2.4. Conventional culturing of brine shrimp

The same *Artemia franciscana* cysts as in the gnotobiotic setup were used in the conventional experiments. Cysts were hatched in salt water with strong aeration and constant illumination at 28°C during 18-22 h. Groups of 20 larvae (instar II stage nauplii) were collected and transferred to glass tubes with salt water for the duration of the challenge test. The same sampling points as for the gnotobiotic cultures were kept for the conventional cultures. Each treatment consisted out of 4 replicates.

4.2.5. Total length of *Artemia franciscana*

At each sampling point 5 live *Artemia* were fixed with Lugol's solution to measure their individual length, using a dissecting microscope equipped with a drawing mirror, a digital plan measure and the software *Artemia* 1.0® (courtesy of Marnix Van Damme).

4.2.6. Histological analysis

Nauplii for light microscopy analyses were fixed and processed according to the procedure described by Gunasekara *et al*. (2011). Samples were fixed for a short period of time to minimise shrinkage followed by immediate processing of the samples. The nauplii were fixed for 5 min in a fixative consisting of 80 ml of 100% ethyl alcohol, 15 ml of 40% formaldehyde and 5ml of acetic acid and subsequently transferred to 70% ethanol. After pre-staining with haematoxylin (Haematoxylin (C.I. 75290), Merck KGaA, Darmstadt, Germany), each nauplius was orientated in 1.5–2.0% agarose (electrophoresis grade GIBCOBRL, 15510-019, Life technologies, Paisley, Scotland) and dehydrated using a tissue processor (STP 420D, Microm International GmbH**,** Thermo Fisher Scientific, Waldorf, Germany) for approximately 22 h, followed by embedding in paraffin using an embedding station (EC350-1 and 350- 2, Microm International).

Per sampling point and treatment (gnotobiotic/conventional) 5 nauplii were cut into serial dorsoventral sections with a thickness of 5 µm using a microtome (HM360, Microm International). All histological sections were stained with haematoxylin and eosin (Eosin yellow (C.I. 45380), VWR International bvba/sprl, Leuven, Belgium). The histological sections were examined under a motorised light microscope (Olympus BX61, Olympus Belgium, Aartselaar, Belgium) linked to a digital camera (Olympus DP 50, Olympus Belgium).

4.2.7. Morphometry

Morphometrical analyses were performed in order to show whether a bacteria-free and conventional environment have the same effect on the development of the alimentary tract of *Artemia* nauplii. Measurements of the height of the gastro epithelial cells from the foregut, midgut and hindgut were performed, as well as on the *microvilli* brush border from the midgut. Five nauplii were collected per treatment (gnotobiotic vs. conventional) at day 2 and day 4 for light microscopy analysis and cut into serial dorsoventral sections of 5 µm. Per nauplius 10-15 sections of the gastrointestinal tract were examined using the Olympus BX61 microscope and Cell D software (Soft Imaging System, Olympus NV) depending on the size of the nauplii (day 2 vs. day 4 respectively).

4.2.8. Transmission electron microscopy

For transmission electron microscopy, a similar protocol was used as described by De Spiegelaere *et al*. (2008). Five nauplii were collected at day 2 and day 4 per treatment (gnotobiotic vs. conventional) for transmission electron microscopy. Samples were fixed in Karnovsky's fixative overnight at 4°C, after which specimens were washed in a sodium cacodylate buffer (pH 7.4), and post fixed overnight in 1% osmium tetroxide. Subsequently, the samples were dehydrated using a graded alcohol series (50% to 100%) and finally embedded in Spurrs' resin. Per sampling point and treatment, 5 nauplii were cut into semi-thin sections. After examination of these semi-thin sections to localise the regions of interest, ultra-thin sections of 60 nm were made using a Leica EM MZ 6 ultramicrotome (Leica Mircosystems GmbH), and mounted on formvar coated single slot copper grids (Laborimpex N.V., Brussels, Belgium). 10 ultra-thin sections per animal were made and studied. The sections were post-stained with uranyl acetate and lead citrate (Leica EM Stain, Leica Microsystems GmbH) and viewed on a Jeol 1400 plus TEM (JEOL Ltd., Tokyo, Japan) at 80 kV accelerating voltage.

4.2.9. Statistical analysis

Collected data for individual length and histological measurements were analysed using a one-way analysis of variances (ANOVA) with Tukey's post hoc test. Homogeneity of variances and normality of data were fulfilled. All statistical analyses were done using the Statistical Package for the Social Sciences (SPSS), version 21.0 using a significance level of 5%.

4.3. RESULTS

4.3.1. Total length of *Artemia franciscana*

In a first experiment, the total length of the cultured animals was measured to determine the difference between gnotobiotically reared nauplii versus conventionally reared animals (Figure 4.2). At day 2 no significant differences were observed between both groups (2.0 ± 0.5 mm for both groups). However, at day 4 the total length of the conventionally reared animals had increased (3.5 ± 0.8 mm) by more than 50% compared to the gnotobiotic animals (2.0 ± 0.5 mm).

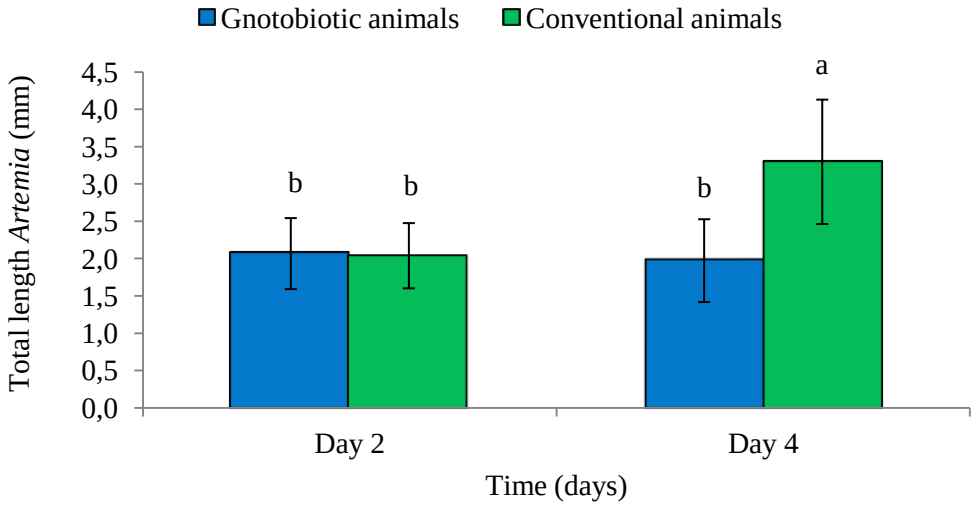

Figure 4.2: Total length of conventionally and gnotobiotically reared brine shrimp at day 2 & day 4 (Average ± standard deviation of five replicates). Bars with different letters are significantly different ($P < 0.05$).

4.3.2. General morphology of the foregut, midgut and hindgut

The foregut of *Artemia* is lined with cuboidal cells and no microvilli brush border is present on top of the epithelial cells. The surface of the epithelial cells is covered by a thin cuticle layer (Figure 4.3A). There is a clear transition from cuboidal to cuboidal-columnar cells from the foregut epithelial cells to the midgut epithelial cells (Figure 4.3B). There are two gastric caeca in the gastrointestinal tract where the foregut transitions into the midgut, lined with the same epithelial cells as can be found in the midgut section (Figure 4.3A). The midgut is lined with columnar-cuboidal epithelial cells that are covered by a brush border of microvilli (Figure 4.3). Histological sections of gnotobiotically and conventionally reared animals were rather similar at day 2. For both groups a small microvilli brush border was visible. At day 4 however, a significant difference between the two becomes obvious. The microvilli brush border of the conventional animals became clearly visible at day 4 (Figure 4.3E) and the cells are neatly aligned next to each other (Figure 4.3D). Some of the gnotobiotic animals that were studied showed epithelial cells in the midgut that were very similar to the ones observed for the conventional animals. Yet most of times the gnotobiotic *Artemia* showed an underdeveloped midgut section with almost no brush border present or a

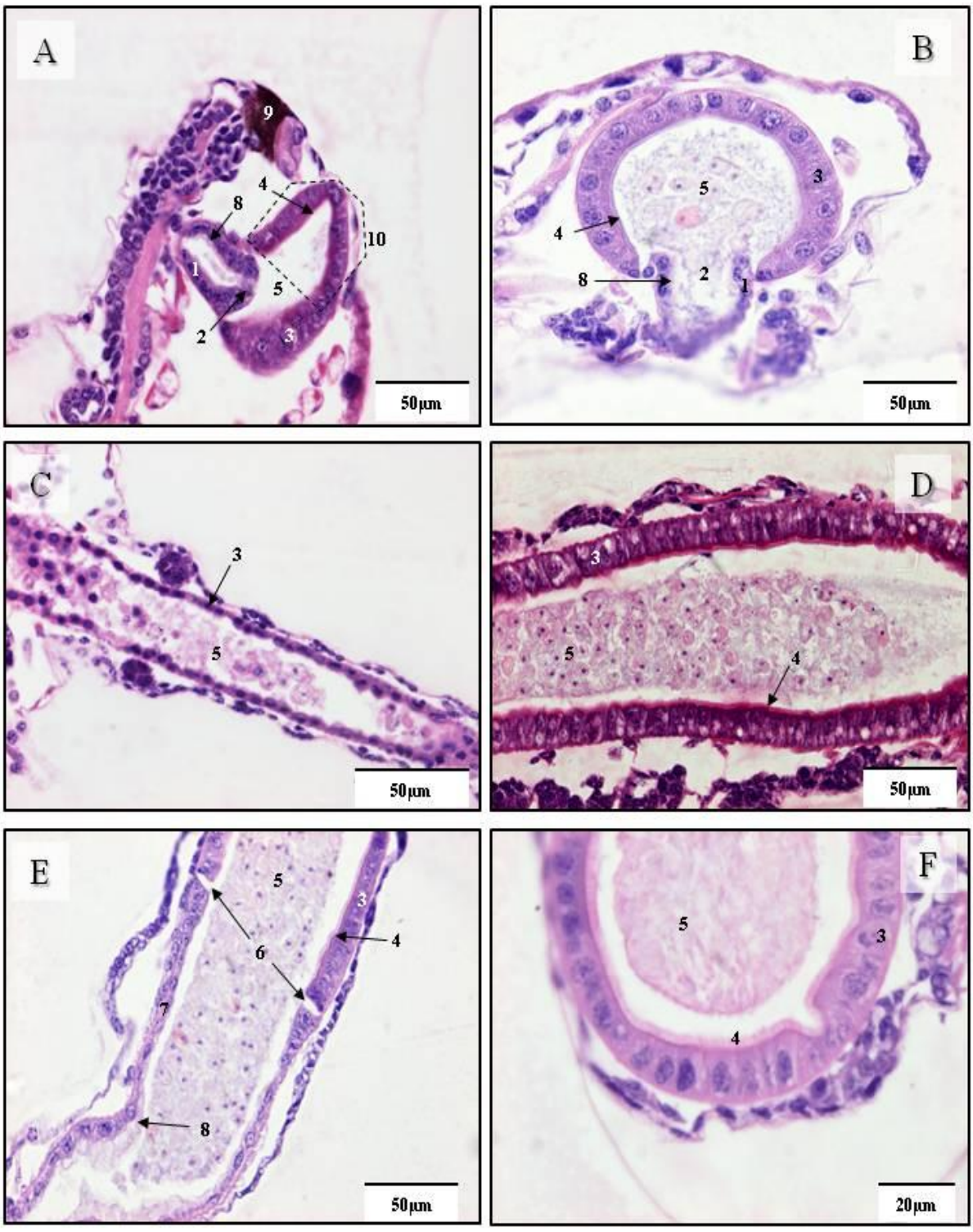

Figure 4.3: Histological sections of the alimentary tract of *Artemia franciscana*. **A**: Longitudinal section of the foregut of 2-day old gnotobiotic *Artemia*; **B**: Transversal section of the foregut-midgut transition of 2-day old gnotobiotic *Artemia*; **C**: Longitudinal section of the midgut of 4-day old gnotobiotic *Artemia*; **D:** Longitudinal section of the midgut of 4-day old conventional *Artemia*; **E**: Longitudinal section of the midgut-hindgut transition of 4-day old conventional *Artemia*; **F**: Detail of the microvilli brush border in the midgut of 4-day old conventional*Artemia*. **Legend: 1** foregut cells; **2** foregut-midgut transition; **3** midgut epithelial cells; **4** brush border; **5** gut lumen; **6** midgut-hindgut transition; **7** hindgut cells; **8** cuticular layer; **9** naupliar eye; **10** caeca

very short one. The arrangement of the epithelial cells has become more chaotic and the overall condition of the midgut has deteriorated (Figure 4.3C).

The transition from midgut to hindgut is clearly visible, together with another shift from columnar-cuboidal to a cuboidal epithelium (Figure 4.3F). The length of the hindgut is shorter compared to the midgut. The hindgut consists out of cuboidal cells without the apical microvilli that are present in the midgut. A cuticular layer covers the epithelium cells instead. Cell height of the hindgut cells was overall higher in the conventional animals compared to the gnotobiotic animals.

4.3.3. Cell height of the epithelial cells of the foregut, midgut and hindgut

The total height of epithelial cells lining the gastrointestinal tract of *Artemia* nauplii was measured for both gnotobiotic and conventionally reared nauplii at day 2 and day 4 of the experiment (Figure 4.4). Measurements were obtained for the foregut, midgut and hindgut for both groups of cultured brine shrimp per day. The cell height of the foregut did not significantly differ between conventional and gnotobiotic animals at day 2. Neither were there any significant differences in the cell height at day 4. However, there was a significant decrease in cell height of the foregut epithelial cells of gnotobiotic animals from day 2 to day 4 (6.0 ± 1.0 µm at day 2 and 4.5 ± 1.0 µm at day 4).

Major differences were observed for the midgut (Figure 4.4). For the gnotobiotic animals observations at day 2 and day 4 were similar. There was no increase in cell height of the epithelial cells of the midgut in gnotobiotic animals. A slight decrease, though not significant, could be observed: 8.5 ± 1.5 µm at day 2 and 7.0 ± 1.0 µm at day 4. Overall cell height of the midgut epithelial cells was significantly higher for conventional animals compared to the gnotobiotic animals. This significant difference in the height of the midgut epithelial cells between gnotobiotic and conventional animals was present from day 2. For the conventional animals a significant increase from 13 ± 2.0 µm to 15.5 ± 4.0 µm occurred from day 2 to day 4.

A significant difference was also observed for the hindgut. Overall cell height of the epithelial cells in the hindgut was significantly lower for gnotobiotic nauplii (5 ± 0.5 µm at day 2 and 4.0 ± 1.0 µm at day 4) compared to conventional larvae (6.5 ± 1.5 µm at day 2 and

6 ± 1.5 µm at day 4). Yet, cell height did not change over time for each respective group (gnotobiotic/conventional) (Figure 4.4).

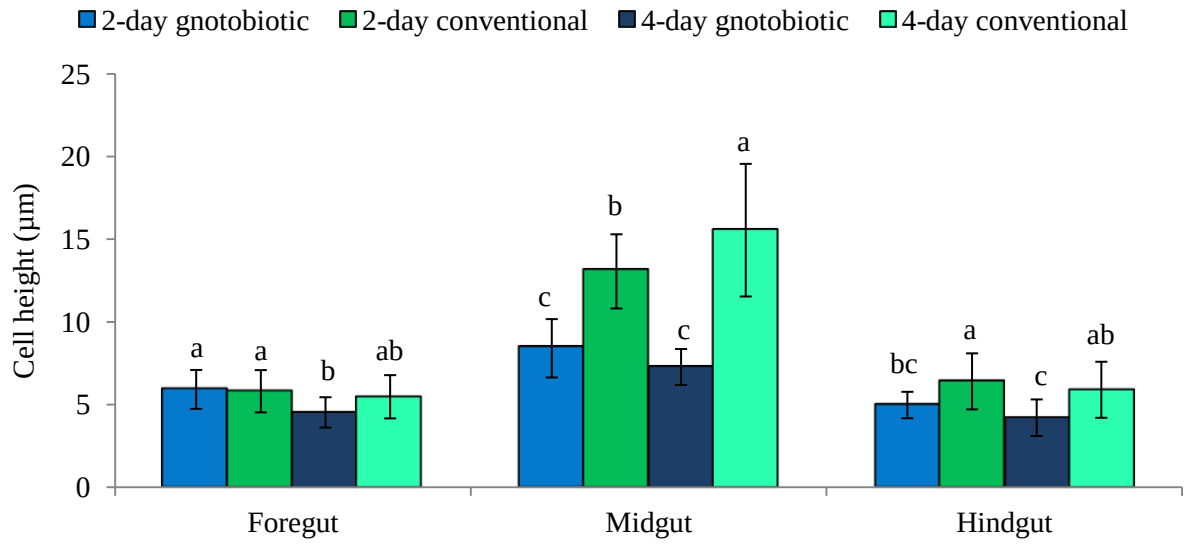

Figure 4.4: Height of the epithelial cells of the foregut, midgut and hindgut of conventionally reared brine shrimp versus gnotobiotically reared nauplii for day 2 and day 4. (Average ± standard deviation of five replicates). Bars with different letters are significantly different for each respective section of the GI of the brine shrimp ($P < 0.05$).

4.3.4. Microvilli brush border of the midgut

A closer look at the brush border present on the epithelial cells in the midgut showed that there was an important difference between the gnotobiotic animals and the conventionally reared ones. Results showed that the brush border of conventional animals was significantly higher than the one from gnotobiotic brine shrimp. The brush border of conventional nauplii is at least double the height of the one observed in gnotobiotic animals (Figure 4.5). This significant difference in height was already present from day 2 between both groups of animals. A significant increase (from 1.81 ± 0.41 µm to 2.20 ± 0.47 µm) could be observed for the brush border of conventional nauplii from day 2 to day 4. No such increase was observed for the brush border of gnotobiotic animals during the same time frame. A slight decrease (from 0.95 ± 0.25 µm to 0.90 ± 0.28 µm) occurred over time for the gnotobiotic animals, although not statistically significant.

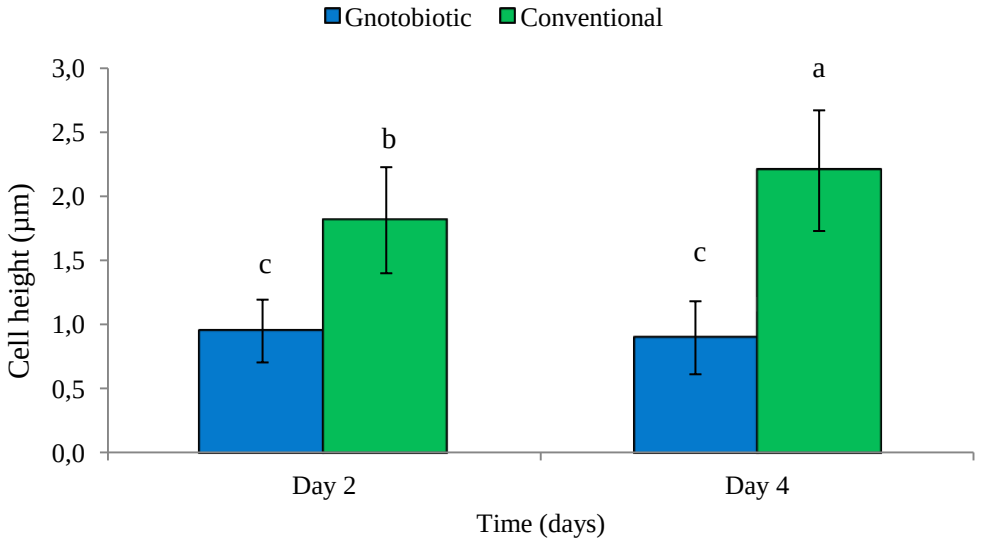

Figure 4.5: Height of the microvilli of the midgut. Conventionally reared brine shrimp versus gnotobiotically reared nauplii (day 2 - day 4). (Average ± standard deviation of five replicates). Bars with different letters are significantly different ($P < 0.05$).

4.3.5. Transmission electron microscopic (TEM) observations

TEM was performed to further investigate the gastrointestinal tract of *Artemia franciscana*, especially the microvilli brush border of the midgut section (Figure 4.6). Light microscopy revealed that the brush border of the midgut epithelium was significantly underdeveloped in the gnotobiotic animals at day 4 (Figure 4.3), which was confirmed by height measurements of the microvilli (Figure 4.5).

Three things become apparent when looking at the transmission electron microscopy sections. First of all, there are less microvilli present in the midgut of the gnotobiotic animals, both for day 2 and day 4 compared to the conventional animals (Figure 4.6 A&B). This difference becomes increasingly clear at day 4 (Figure 4.6 C&D). The conventional animals thrive and the brush border grows over time. For the axenic animals, the microvilli were becoming shorter or absent over the sampling time (Figure 4.6E). Secondly, next to the absence of the microvilli, the cells become more irregular, cell organelles and nuclei become less abundant and disruption of the cell membranes at the apical part of the cell was observed for the gnotobiotic animals over time (Figure 4.6C).

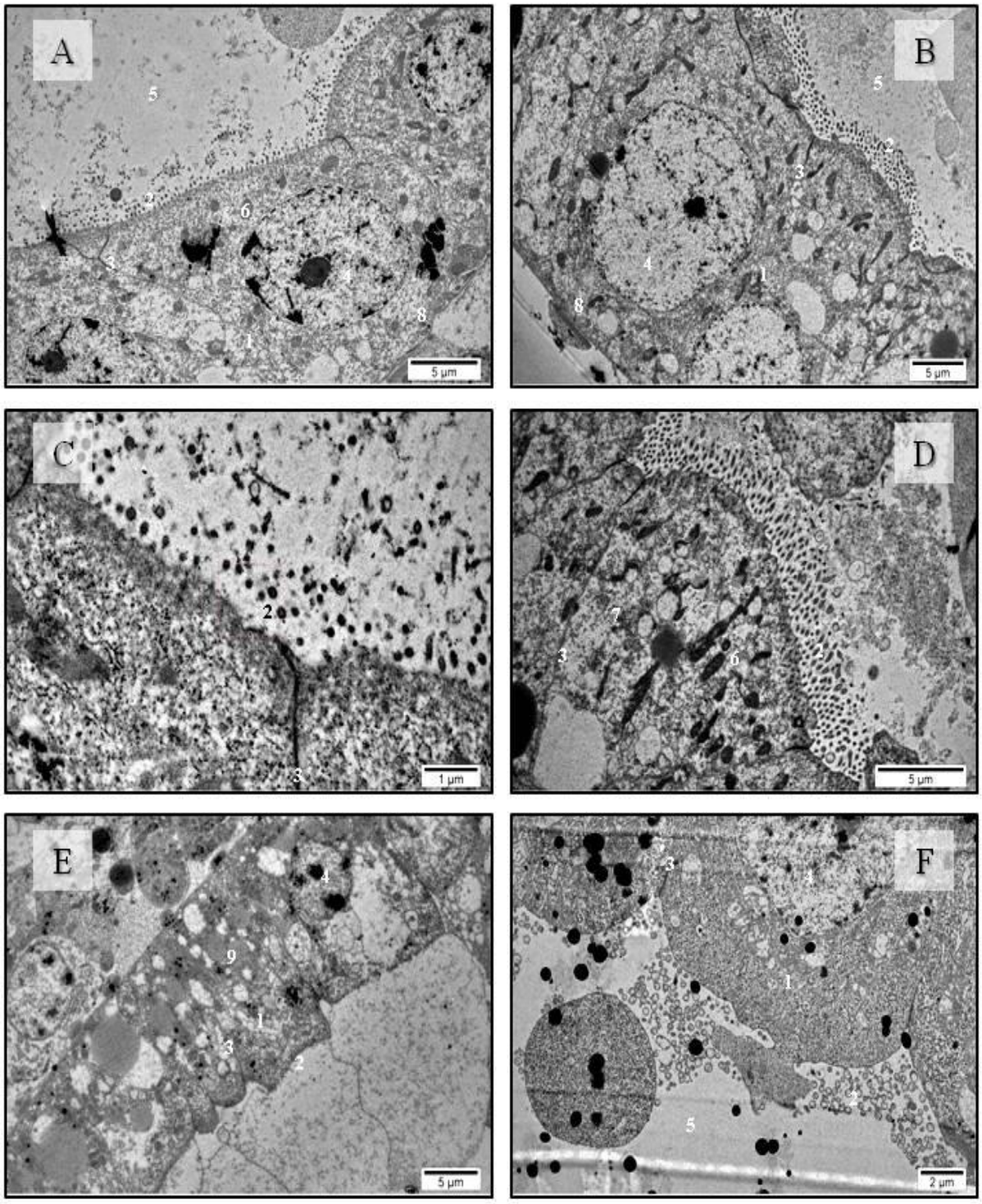

Figure 4.6: Transmission electron micrographs of the midgut of *Artemia franciscana* fed with axenic microalgae *Tetraselmis seucica*. **A**: Midgut epithelial cell of 2-day old gnotobiotic animals; **B**: Midgut epithelial cell of 2-day old conventionally reared animals; **C**: Detail of the microvilli brush border of 2-day old gnotobiotic brine shrimp; **D**: Detail of the microvilli brush border of 2-day old conventional brine shrimp; **E**: Midgut enterocyte of 4-day old gnotobiotic animals; **F**: Midgut enterocyte of 4-day old conventionally reared animals. **Legend: 1** midgut enterocytes; **2** microvilli; **3** junctional complex; **4** nucleus; **5** midgut lumen; **6** mitochondria; **7** endoplasmatic reticulum; **8** basal infoldings; **9** lysosomes

The cellular junctional complexes remain intact over time though (Figure 4.6). Cell organelles are more abundantly present in conventional animals. The cytoplasm of the midgut epithelial cells contains numerous mitochondria and a well-developed endoplasmic reticulum. Large central nuclei are present throughout the whole midgut epithelium (Figure 4.6D). Finally, at day 4, in the midgut epithelial cells of the gnotobiotic animals the occurrence of large vacuoles can be observed.

4.4. DISCUSSION

During a normal life cycle, animals come into contact with a huge variety of micro-organisms. Some of these microbes are harmful to the host animals; although a lot of them are beneficial for the development of animals during their life cycle (Rawls *et al*., 2004). These microbial communities are sometimes eliminated to obtain gnotobiotic conditions in order to better understand the function of the host animal. Gnotobiotic conditions allow for a better control of variables and reproducibility of experiments (Pleasants, 1973). The idea behind it is that in the absence of the micro-organisms the functioning of the host animal would be elucidated. The difficulty is that these gnotobiotic animals are more often underdeveloped both in morphology as in immune functioning. Before making any extrapolations of obtained results, data should always be confirmed in conventional systems (Gordon and Pesti, 1971).

Artemia franciscana are often used as gnotobiotic model systems for marine invertebrates. The aim of this study was to evaluate axenic microalgae as a feed to obtain adult gnotobiotic animals. This was done by making a comparative study of the histology and cellular morphology of the alimentary tract of gnotobiotic *Artemia* nauplii compared to their conventional counterparts. The focus lay on the early stages of the life cycle of *Artemia*, since the gnotobiotic animals did not survive past day 5. The axenic microalgae did not allow for *Artemia franciscana* growing up until adulthood in the gnotobiotic system. Another feed needs to be identified if one wants to observe the maturation of the GI tract in gnotobiotic *Artemia* until adult. The comparison of the GI tract of gnotobiotic and conventionally grown animals made in this study revealed severe deficiencies in the morphology of the gnotobiotic *Artemia* already in the early stages of their life.

The increase in cell height observed for the midgut and hindgut of conventional animals compared to gnotobiotic animals, can be explained by the presence of live bacteria in the conventional system. Previous studies on mice, rats and zebrafish have shown that beneficial bacteria increase cell proliferation rates and cell kinetics of the epithelial cells (Banasaz *et al*., 2001 & 2002; Abrams *et al*., 1963; Uribe *et al*., 1997; Rawls *et al*., 2004). Next to this, these bacteria can provide an extra direct feed source for *Artemia franciscana* nauplii. They are also capable of inducing the production of digestive enzymes in the gastrointestinal tract of *Artemia franciscana* and the recycling of nutrients. Finally, bacteria improve the water quality by removing toxic metabolites (Rieper, 1978; Verschuere *et al*., 2000). These can possibly affect the growth and survival of the nauplii, of which the gnotobiotic animals in this study could not benefit. For *Artemia*, previous studies have already showed the critical role of bacteria and their nutritional value for growing *Artemia franciscana* (D'Agostino, 1980; Intriago & Jones, 1993).

At day 2, the internal development of the animals is comparable between both groups, both on cell height, microvilli brush border and cell organelles present in the cells. On the contrary a deteriorated state of the gastrointestinal tract of the gnotobiotic animals was clearly visible at day 4. Less cell organelles, more vacuolisation and fewer microvilli in the brush border are clear indications that the cell morphology has been disturbed. This can point to the direction of autophagy (Rekecki, 2012). Autophagy is the phenomenon of a basic cellular mechanism involved in cell degradation of redundant cellular components during starvation or under physiological stress (Kuma *et al*., 2004; Olsen *et al*., 2008). The brush border is important given that it has an absorptive function as described by Kikuchi (1971) in conventional adult *Artemia*. Mitochondria in the epithelial cell lining and infoldings of the basal cellular surfaces are indicative of the absorptive function of these cells (Hootman and Conte, 1974; Gunasekara *et al*., 2011). The amount of mitochondria in cells varies on the basis of its energy requirements. They are most commonly found in the apical part of the cell, close to the microvilli brush border, where most of the energy is consumed. Since the microvilli brush border and the cell organelles were reduced at day 4 for gnotobiotic animals, we can hypothesise that the gnotobiotic animals did not succeed in absorbing sufficient nutrients to maintain their homeostasis. Gnotobiotic animals might retreat to this basic survival strategy at day 4. As a result one should be careful in using these animals for further host-microbe interaction studies. As animals grow older they can suffer from 'wasting'

syndrome with symptoms as weight loss, muscle atrophy and fatigue. Further research is warranted to establish this phenomenon in gnotobiotic *Artemia franciscana* though.

4.5. CONCLUSION

In this study, we characterised the morphological alterations occurring in the gastrointestinal tract of *Artemia franciscana* animals over a time period of 4 days for conventional and gnotobiotical animals. Results showed minor differences between both groups at day 2, however around day 4 the gnotobiotic animals are clearly underdeveloped. This can be seen in the total length of the animals as well as in the development of the epithelial cells and the brush border of the gastrointestinal tract. In conclusion, the gut integrity was well preserved for nauplii fed with axenic microalgae at day 2, however no such conclusion can be made for the axenic feeding as animals grow older

CHAPTER 5

CAN CHANGES IN THE MORPHOLOGY OF THE GASTROINTESTINAL TRACT EXPLAIN AN INCREASE IN SURVIVAL UPON INFECTION WITH *VIBRIO*?

A MORPHOLOGICAL STUDY OF *ARTEMIA FRANCISCANA*

In collaboration with Prof. dr. ir Peter Bossier and Prof. dr. Wim Van Den Broeck

CHAPTER 5:

Can changes in the morphology of the gastrointestinal tract explain an increase in survival upon infection with *Vibrio*?

A morphological study of *Artemia franciscana*

ABSTRACT

When a pathogenic bacterium wants to invade its host animal, several steps are involved. The infectious cycle includes entry into the host, establishment and multiplication together with damage to host tissues and cells. Several protective barriers against bacteria are in place in the host animal, for crustaceans one of these is for example the exoskeleton. The acid mucins in the gastrointestinal tract are another well-known barrier for different animals. Cells responsible for the mucin production are known as goblet cells. Several studies, e.g. in sea bass, showed that the appearance of these cells only occur several days after hatching. This is correlated with increased protection against pathogens after a certain period of time. During a series of challenge tests with conventionally grown *Artemia franciscana* (fed with *Tetraselmis suecica* 66/4) an increase in survival was observed when exposed to a *Vibrio* challenge occurring around day 6 after hatching. The hypothesis was that *Artemia* nauplii may not be able to count on the various defence mechanisms in the early stages of development that are in place for older animals. Histological analyses were done using different cellular stainings and cell measurements. Morphological alterations in the gastrointestinal tract from day 2 until day 8 were identified in order to find a possible explanation. Results showed the appearance of goblet cells around day 6 with no clear visible presence of them before this day. Yet no other stainings showed any significant morphological changes occurring over the experimental period. The epithelial cell height and the microvilli brush border did gradually increase over time. Based on the obtained results in this study we could hypothesise that the occurrence of goblet cells might provide an explanation for the observed changes in survival. Further research is warranted though to elucidate these changes in morphology and the mechanisms involved providing protection for these animals against pathogens.

5.1. INTRODUCTION

Previous infection studies on sea bass and *Artemia franciscana* showed changes in survival after a *Vibrio* exposure as the animal gets older (Gunasekara *et al.,* 2011; Rekecki, 2012). Gunasekara *et al.* (2011) observed an increase in the survival of *Artemia franciscana* after a *Vibrio* challenge from DAH6 onwards; however no explanation was found. In this study, an increase in survival was also observed around day 6 during challenge tests with conventionally grown *Artemia franciscana* (fed with *Tetraselmis suecica* 66/4) upon a challenge with *Vibrio* sp. H6. It has been hypothesised that this is caused by the occurrence or changes in the composition of the mucus layer or changes in the surrounding connective tissue or the basal membrane. It could also be due to the formation of a peritrophic membrane in crustaceans (Martin *et al*., 2006; Gunasekara *et al.*, 2011; Rekecki, 2012). For sea bass the presence of mucins in the digestive tract is of particular importance as a defence barrier (Rekecki, 2012). Changes in the composition of the mucus layer may change the ability of the host animal to defend itself against pathogens. Acid mucins for example, cannot be degraded as much by bacterial glycosidases and host proteases, therefore providing a protective barrier against bacteria in the gastrointestinal tract of animals (Roberton & Wright, 1997). Several studies showed that goblet cells are responsible for this acid mucin production. In sea bass they only appeared between 6 days (Rekecki, 2012) and 11 days after hatching according to studies done by Micale (2006).

The aim of this study was to characterise internal morphological developments in conventional *Artemia franciscana* that might explain the change in survival around day 6 after a challenge with *Vibrio* sp. H6. The hypothesis was that *Artemia* nauplii in the early stages of development may not be able to count on the various defence mechanisms that could be in place for older animals. The epithelial cell height as well as the thickness of the microvilli border was measured to see the general state of the gastrointestinal tract. Changes in the surrounding connective tissue and differences in the basal membrane surrounding the gastrointestinal tract were checked and the presence of goblet cells was verified.

5.2. MATERIAL & METHODS

5.2.1. Bacterial strains & growth conditions

The isolate of *Vibrio campbellii* LMG21363 used in this study as a primary pathogen, was obtained from the *Vibrio* collection in the Laboratory of Aquaculture & *Artemia* Reference Center, Ghent University, Belgium. The isolate H6, belonging to the *V. harveyi* clade (Vanmaele *et al*., 2015), kept at the Laboratory of Aquaculture & *Artemia* Reference Center, Ghent University, Belgium, was used as a second pathogenic strain in the challenge test. The bacterial isolates were previously stored in 30% glycerol at -80° C. They were grown in marine broth (Difco^{TM}) at 28°C with constant agitation. Densities were determined spectrophotometrically at 550 nm and the calculation was done according to the McFarland standard (BioMerieux, Marcy L'Etoile, France), assuming that an OD550=1.000 corresponds to 1.2×10^{9} cells ml^{-1}.

5.2.2. Conventional culturing of brine shrimp & microalgae

Experiments were performed with *Artemia franciscana* cysts, originating from Great Salt Lake, Utah, USA (INVE Aquaculture NV, Belgium) and the San Francisco Bay strain (directly from San Francisco Bay via Jim Clegg). Cysts were hatched in salt water with strong aeration and constant illumination at 28°C during 18 - 22 h. Groups of 20 larvae (instar II stage nauplii) were collected and transferred to glass tubes with salt water for the duration of the challenge test. The animals were fed with *Tetraselmis suecica*, grown in Walne medium (Walne, 1967). All parameters for algal culture were kept constant: pH 7, continuous light of 100 µmol photons m^{-2}s^{-1}, temperature of 24°C and 30 g l^{-1} salinity. The density of the algae culture was measured using a Bürker hemacytometer and the feeding regime was adapted from Marques *et al*. (2004).

5.2.3. Conventional challenge tests with brine shrimp

For the conventional challenge test isolates of the bacterial strains were aseptically inoculated in 30 ml marine broth 2216 (Difco™) and incubated overnight at 25°-28°C with constant agitation. 150 µl was subsequently transferred and grown to stationery phase in 30 ml marine broth 6 h before challenge. The challenge test was performed according to Marques *et al*. (2006). The bacterial suspension was added to reach a final density of 10^{7} cells ml^{-1}. Each treatment consisted out of 4 replicates.

The survival of the brine shrimp larvae was determined after 48 h according to Amend (1981). The relative percentage of survival (RPS) was calculated as follows:
RPS (%) = (% of surviving challenged larvae)/ (% of surviving unchallenged larvae) x 100

Three groups were kept during the infection study: Group 1 was challenged with *Vibrio campbellii* LMG21363, Group 2 was challenged with *Vibrio* sp. H6 and Group 3 was not exposed to a challenge and kept as a control. Survival was counted for all three groups, but samples for histological analysis were taken only from the control group which was not exposed to challenge (Group 3). Ten animals were collected every two days and sampling points were at day 2, day 4, day 6 and day 8.

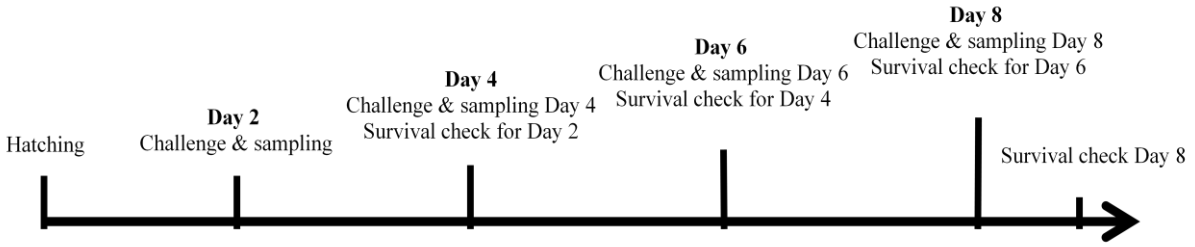

Figure 5.1: Timeline for the experimental design of this chapter.

5.2.4. Histological analysis

Nauplii for light microscopy and histological analyses were fixed and processed according to the procedure described by Gunasekara *et al*. (2011). The nauplii were fixed for 5 min in a fixative consisting of 80 ml of 100% ethyl alcohol, 15 ml of 40% formaldehyde and 5 ml of acetic acid and subsequently transferred to 70% ethanol. After pre-staining with haematoxylin (Haematoxylin (C.I. 75290), Merck KGaA, Darmstadt, Germany), each nauplius was orientated in 1.5–2.0% agarose (electrophoresis grade GIBCOBRL, 15510-019, Life technologies, Paisley, Scotland) and dehydrated using a tissue processor (STP 420D, Microm International GmbH**,** Thermo Fisher Scientific, Waldorf, Germany) for approximately 22 h, followed by embedding them in paraffin using an embedding station (EC350-1 and 350- 2, Microm International).

Per treatment and per day, 10 nauplii were cut into serial transverse sections of 5 µm thickness using a microtome (HM360, Microm International). For general histology, the sections were stained with haematoxylin (Haematoxylin (C.I. 75290), Merck KGaA,

Darmstadt, Germany) and eosin (Eosin yellow (C.I. 45380), VWR International bvba/sprl, Leuven, Belgium). The sections were also stained with Van Gieson, Masson's trichrome, a reticulin staining and Alcian blue-periodic acid-Schiff staining (AB-PAS) at pH 2.5 (Mowry and Winkler, 1956). The histological sections were examined and photographed using a motorised microscope (Olympus BX 61, Olympus Belgium, Aartselaar, Belgium) linked to a digital camera (Olympus DP 50, Olympus Belgium).

5.2.5. Morphometry

Morphometrical analysis was performed on the height of the gastro epithelial cells from the foregut, midgut and hindgut, as well as on the microvilli brush border from the midgut. Ten nauplii were collected per treatment (gnotobiotic vs. conventional) at day 2, 4, 6 and 8 for light microscopy analysis and cut into serial dorsoventral sections of 5 µm. Per nauplii 10 sections of the gastrointestinal tract were examined using the Olympus BX61 microscope and Cell D software (Soft Imaging System, Olympus NV).

5.2.6. Statistical analysis

Survival data and the histological measurements were subjected to a one-way analysis of variances (ANOVA) with Tukey's post hoc test. Homogeneity of variances and normality of data were fulfilled. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 21.0 using a significance level of 5%.

5.3. RESULTS & DISCUSSION

5.3.1. Challenge tests with conventional brine shrimp

During a series of challenge tests at day 2, day 4, day 6 and day 8, a significant increase in survival percentages of *Artemia franciscana* was observed occurring around day 6 (Figure 5.2). *Vibrio* sp. H6 and *Vibrio campbellii* LMG 21363 were used as pathogens towards conventionally reared brine shrimp larvae. Challenge with H6 showed the lowest survival during the experimental period with very low survival at the first two sampling points, 0% and 5% survival at day 2 and day 4, respectively. For both H6 and *Vibrio campbellii* LMG 21363, survival was significantly lower compared to the control group up to day 2 and day 4.

Starting from day 6, survival is no longer significantly different from the control group. The same was true for survival observed at day 8 for all experimental treatments.

The experiment was repeated using a different strain of *Artemia franciscana* originating from San Francisco Bay. This was done in order to check whether or not the observed mortality rates were similar when using a different strain of *Artemia*. In these tests animals going from 2-day old nauplii up to 3 weeks old animals were used. Results showed the same trend as shown in this study (data of these tests are not shown here). After day 6, no significant differences in survival were observed between the control group and both experimental treatments.

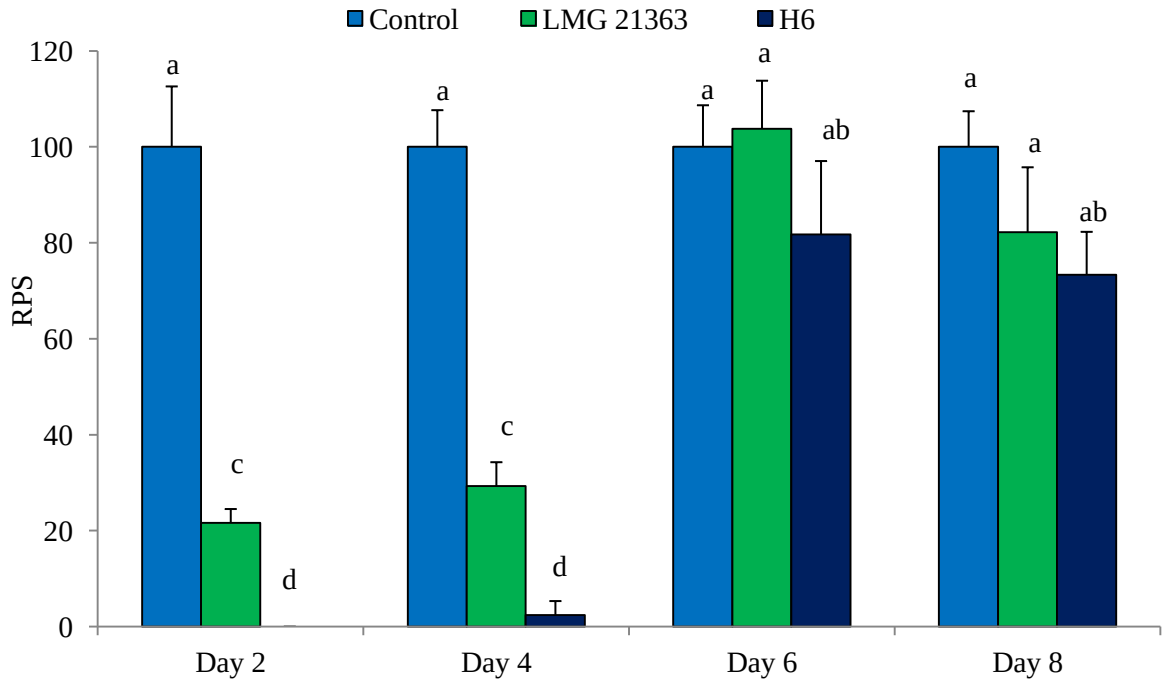

Figure 5.2: Average survival % of conventionally reared brine shrimp larvae 48h after challenge with the *Vibrio* sp. H6 and the pathogen *Vibrio campbellii* LMG 21363 at day 2, day 4, day 6 and day 8 after hatching. Error bars represent the standard deviation of 4 replicates. Bars with different letters are significantly different ($P < 0.05$). The control refers to animals that were not challenged, but otherwise treated in the same way as in the other treatments.

5.3.2. Cell height & microvilli brush border

The epithelial cell height as well as the thickness of the microvilli border was measured during the experimental time frame. A gradual overall increase was observed for both parameters, the highest significant differences were observed at day 8 for both the height of the epithelial cells as well as for the brush border (Figure 5.3). No significant differences in the height of the microvilli border were observed from day 2 until day 6 however.

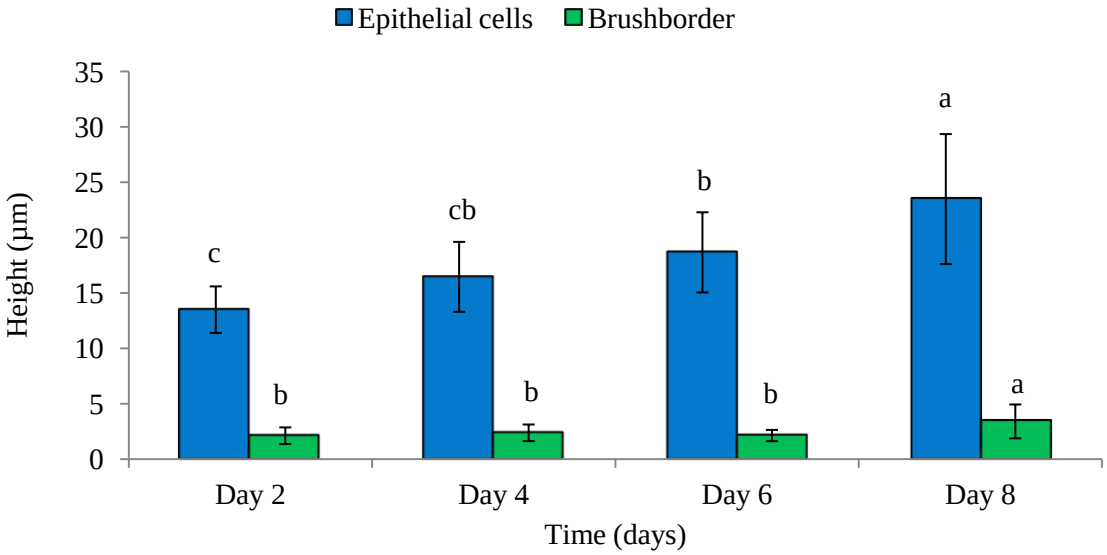

Figure 5.3: Average height (µm) of the epithelial cells of the midgut and the microvilli (brush border) of conventionally reared brine shrimp larvae at day 2, day 4, day 6 and day 8 after hatching. Error bars represent the standard deviation of 5 replicates. Bars with different letters or asterisks are significantly different for each respective part of the cells of the midgut of the brine shrimp ($P < 0.05$).

5.3.3. Histological stainings

To determine whether morphological changes could account for the observed increase in survival around day 6 after a challenge with *Vibrio* sp. H6, different stainings were performed on the histological sections of the animals. A haematoxiline-eosine (HE) staining was done to establish the general state of the alimentary tract. A Masson's trichrome staining and a Van Gieson staining were performed to see changes in the surrounding connective tissue. A reticulin staining was done to check for differences in the basal membrane surrounding the gastrointestinal tract. The Alcian blue- and periodic acid-Schiff (AB-PAS and PAS) staining was performed to identify the presence of goblet cells.

The hematoxylin & eosin (HE) staining makes the cell nuclei, which are basophil, stain blue and the acidofilic, or eosinophilic, structures stain red including most of the cytoplasm and proteins. The HE stained histological sections showed a nicely developed microvilli border for the animals at all sampling points (Figure 5.3 A-D). As the animals grow older more vacuoles occur in the cells and cell height increases. At day 8, goblet cells are visible with the HE staining, though not yet at day 6.

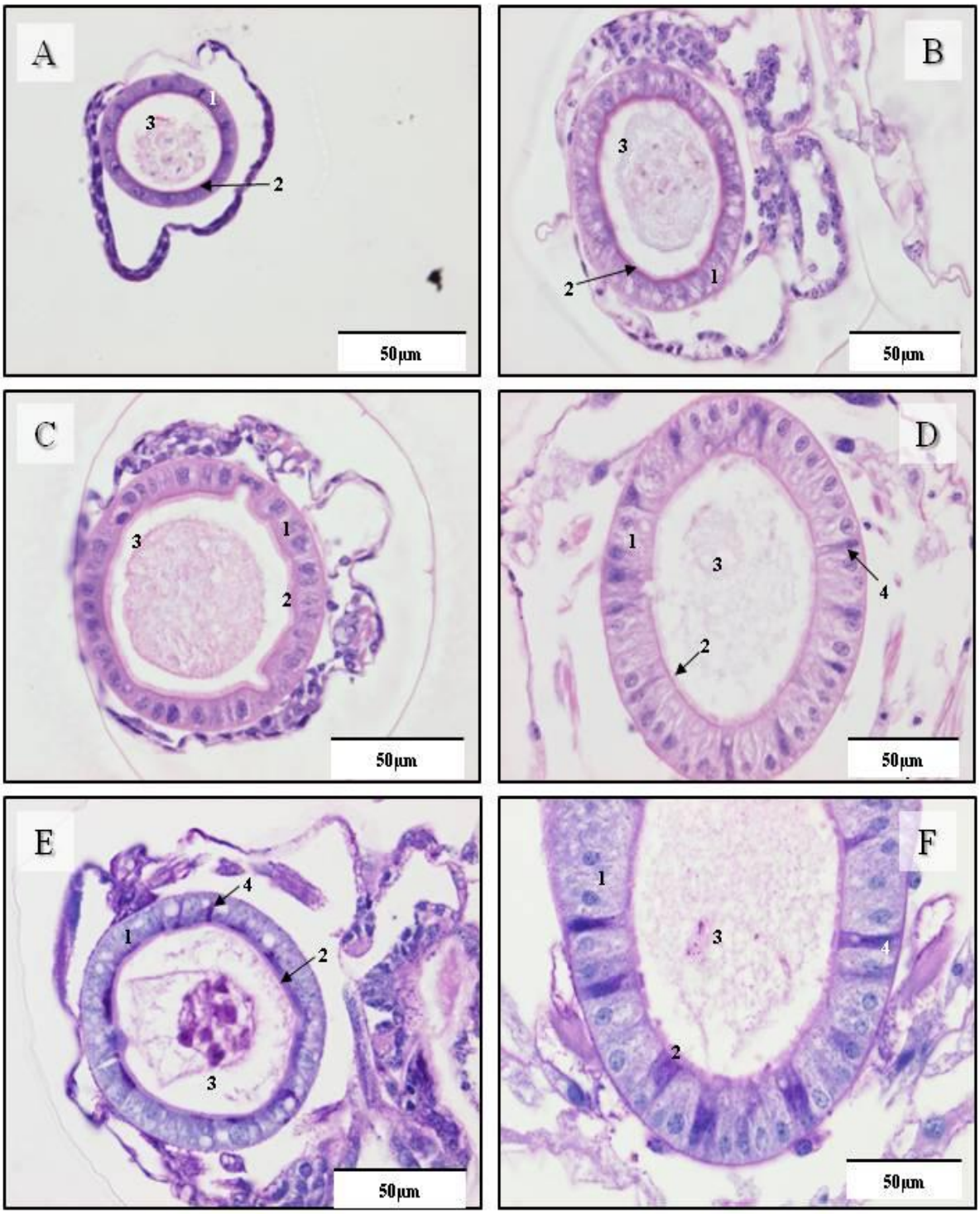

Figure 5.4: A: transversal section of the midgut of 2-day old *Artemia franciscana* with HE staining; **B**: Transversal section of the midgut of 4-day old *Artemia franciscana* with HE staining; **C**: Transversal section of the midgut of 6-day old *Artemia franciscana* with HE staining; **D**: Transversal section of the midgut of 8-day old *Artemia franciscana* with HE staining; **E:** Transversal section of the midgut of 6-day old *Artemia* with PAS staining; **F**: Transversal section of the midgut of 8-day old *Artemia* with PAS staining. **Legend: 1** midgut epithelial cell; **2** brush border; **3** gut lumen**; 4** goblet cell

The periodic acid-Schiff (PAS) staining is used to detect polysaccharides (e.g. glycogen) and mucins. Neutral mucins are colored magenta, acid mucins are blue and cell nuclei are stained blue-violet. Differences occurring over time in the gastrointestinal tract of *Artemia franciscana* could be seen with the PAS staining (Figure 5.4 E&F). The histological sections show the start of the formation of the goblet cells at day 6; and at day 8 the goblet cells are fully present (Figure 5.4 F). The cell nuclei are pressed down to the basal part of the epithelial cell and a goblet cell is formed between two epithelial cells (Figure 5.4 E&F).

For three staining, Masson's trichrome, Van Gieson and reticulin, no different morphological alterations of the gastrointestinal tract were observed in the histological sections at the different days. Masson's trichrome is used to differentiate between cells and their surrounding connective tissue (keratin and muscle fibers color red, collagen blue). Van Gieson's stain is used to distinguish collagen and other connective tissues (nuclei are blue to black, collagen red and muscle, epithelium and erythrocytes yellow). The reticulin staining is used to visualise reticular fibres, brown to black color, and collagen stains light brown to red. No significant changes in connective tissues or reticular fibres are observed in the gastrointestinal tract or in the basal membrane around these days.

The mucosal layer of the alimentary tract is a dynamic environment (Deplancke & Gaskins, 2001). It is responsible for the homeostasis in the host animal and the defence against pathogens coming from the environment. The Alcian blue- and periodic acid-Schiff (AB-PAS) staining is used to make the distinction between acid (AB+) and neutral mucins (PAS+). The staining was performed in this study to see if there were mucosal changes over the experimental period that might help explain changes in the survival of *Artemia franciscana* as was observed by Rekecki and colleagues (2012) in gnotobiotic sea bass larvae. A pH of 2.5 allows to make a difference between neutral (PAS) and acidic mucins (AB), both sulphated and carboxylated (Roberts and Powell, 2003). Acid mucins mean that these mucins have negative charges which reduce the capability of bacteria to bind to them, while these are not present in the neutral mucins. At pH 2.5, mucin of mucous cells stain blue (AB+), purple (PAS/AB+) or magenta (PAS+). The AB-PAS staining however did not show any significant changes in mucus composition in the different phases of development of *Artemia franciscana*. However the mucus layer over the whole sampling period proved to be AB+, indicating that the mucus layer is negatively charged (Figure 5.5).

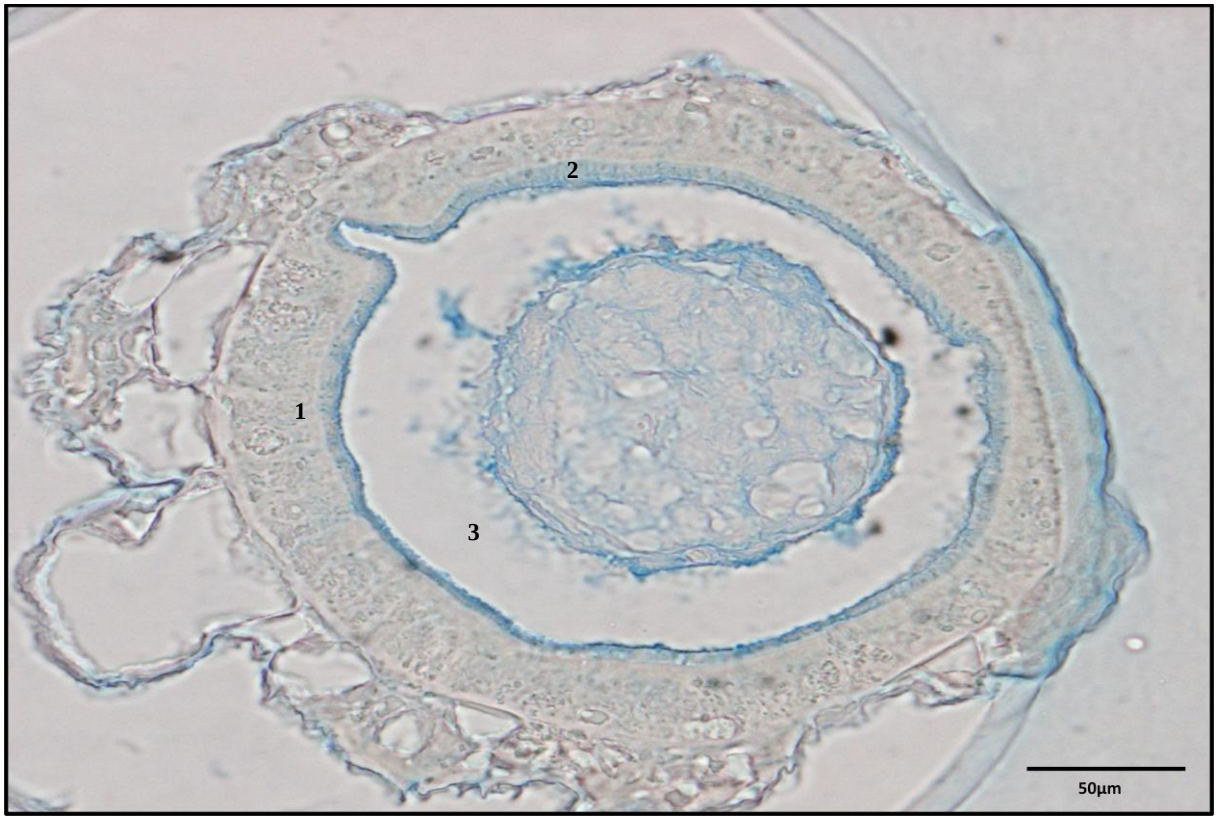

Figure 5.5: Transversal section with AB-PAS staining of the midgut of 6-day old *Artemia franciscana*. **Legend**: **1** midgut epithelial cell; **2** brush border; **3** gut lumen

Another possible explanation for the observed increase in survival could be the formation of a peritrophic membrane in the gut lumen of the animal (PTM). This has been observed in several crustacean species (Martin *et al*., 2006). A peritrophic membrane is an acellular layer that separates ingested items from the midgut epithelium. This layer is usually completely made out of chitin and is only permeable to particles smaller than 20nm. However in this study, the obtained results were not sufficient to elucidate the presence of this PTM. Other fixations and new stainings should be performed to provide an answer to this particular question.

5.4. CONCLUSION

In this study, we characterised the morphological alterations occurring in the gastrointestinal tract of *Artemia franciscana* animals over a time period of 8 days. Results show the appearance of goblet cells around day 6 and no presence of them before this day.

However the other stainings did not show any significant morphological changes over the course of time. Epithelial cell height did increase gradually over time together with the microvilli brush border. Further research is warranted to elucidate other morphological or molecular mechanisms involved in the protection of these animals against pathogens, this in order to fully explain the observed change in survival at day 6.

CHAPTER 6

EFFECT OF β-GLUCANS ON THE SURVIVAL OF GNOTOBIOTIC *ARTEMIA FRANCISCANA* UPON *VIBRIO* INFECTION

In collaboration with Romi Novriadi, dr. Bert Devriendt, Prof. dr Eric Cox, Prof. dr. ir Peter Bossier

CHAPTER 6:

Effect of β-glucans on the survival of gnotobiotic *Artemia franciscana* upon *Vibrio* infection

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ABSTRACT

Invertebrates are believed to lack adaptive immune responses and to only rely on their innate immune mechanisms to battle pathogens. Recent studies however showed that invertebrates might exhibit 'memory-like' immune responses, often referred to as priming. Experimental gnotobiotic conditions have been developed for *Artemia franciscana*, allowing exploring priming activities of different immunostimulants. In this study, the aim was to characterise the survival of gnotobiotic *Artemia* nauplii following a β-1,3/1,6-glucans treatment in combination with an exposure to a virulent *Vibrio* sp. H6. The survival of the animals was monitored upon challenge of gnotobiotic *Artemia* nauplii subsequent to a 6 hour exposure to commercially available β-1,3/1,6-glucan particles (MacroGard) and a washing step. Hence the survival was monitored in the absence of glucan particles. Commercially available β-glucan particles (MacroGard) were offered to gnotobiotic *Artemia*. Results showed a significant improvement in the survival upon infection with a virulent *Vibrio* strain after an exposure to the β-1,3/1,6-glucans. The results of this study indicate that there was protection of *Artemia* by β-1,3/1,6 glucans against a *Vibrio* exposure and that this protection lasted for 6 h.

6.1. INTRODUCTION

Adaptive immunity requires T and B cells, has memory and specificity and is generally considered to be restricted to vertebrates. Invertebrates are believed to only rely on their innate immune mechanisms to combat pathogens (Cerenius & Söderhäll, 1995; Schulenburg *et al*., 2007). Yet studies showed that invertebrates might exhibit their own way of 'memory-like' immune responses, often referred to as priming (Schmid-Hempel, 2005). A high degree of specificity to microbial compounds has also been discovered in several invertebrates (Moret & Siva-Jothy, 2003; Smith *et al*. 2003; Pham *et al*., 2007). For example, in the woodlouse *Porcellio scaber*, primed with heat-killed bacteria, the hemocytes demonstrated increased phagocytosis of the previously encountered bacterial strain compared to other bacteria (Roth & Kurtz, 2009). These data suggest a specific immunological protection by a single exposure to a low dose of heat-killed bacteria, resembling the phenomenon of vaccination. Inappropriate priming, however, can eventually deplete the immune system of the host animal causing reduced fitness and in time possible death (Hauton & Smith, 2007).

As stated by the United Nations' Food and Agriculture Organization (FAO, 2012), diseases are a major constraint to the development of aquaculture. As the prophylactic use of antibiotics is being phased out in Europe, novel approaches are needed in aquaculture, e.g. immunostimulants and probiotics (Marques *et al*., 2006). Several immunostimulants are known to induce and build up protection against a wide range of diseases (Bachère 2003; Smith *et al*., 2003). β-glucans, one of these immunostimulants, are glucose polymers found in fungi, plants, some bacteria and seaweeds.

In invertebrates, β-glucans induce at least two types of humoral responses: coagulation via tranglutaminases, as well as the activation of the melanisation cascade that will turn prophenoloxidase (proPO) into catalytically active phenoloxidase (PO) (Soltanian *et al*., 2009; Cerenius *et al.*, 2010). The prophenoloxidase (proPO) activating system is important in the crustacean immune system as it is one of the major immune defence mechanisms against several pathogens for invertebrate animals (Cerenius and Söderhäll, 2004). Oral stimulation with these β-glucans improves immune responses and resistance or survival during a subsequent infection with harmful micro-organisms (Jiravanichpaisal *et al.*, 2006; Kurtz & Franz, 2003; Hauton & Smith, 2007).

Gnotobiotic brine shrimp (*Artemia franciscana*) cultures were developed to explore the immunomodulatory capacity of different immunostimulants (Marques *et al.*, 2006; Soltanian, 2007; Baruah, 2012). Marques and colleagues showed that the cell wall composition of the yeast significantly determined the survival of *Artemia* nauplii upon a *Vibrio* exposure. It appeared that a higher concentration or bio-availability of β-glucans provided a better protection of the nauplii against an infection by *Vibrios* (Marques *et al.*, 2006). Studies suggest that these effects can be very much pathogen-host specific and should be verified (Rowley & Powell, 2007; Rowley & Pope, 2012).

In this study, the aim was to characterise the effect of β-1,3/1,6-glucans in combination with a virulent *Vibrio* strain H6 challenge in a gnotobiotic *Artemia* system (Vanmaele *et al.*, 2015). For that purpose, commercially available β-glucan particles, produced from the yeast *Saccharomyces cerevisiae* (MacroGard), were offered to gnotobiotic *Artemia* during a period of 6 h and then removed by washing the animals. The survival of the animals pre-treated in this way was monitored upon a *Vibrio* challenge.

6.2. MATERIALS & METHODS

6.2.1. Bacterial strains and culture conditions

The isolate H6, belonging to the *V. harveyi* clade (Vanmaele *et al*., 2015), was used as pathogenic strain in the challenge test. The dead and autoclaved *Aeromonas* sp. strain LVS3 was used as a food source for the *Artemia franciscana* larvae (Verschuere *et al*., 1999). The bacterial isolate H6 and LVS3 were stored in 30% glycerol at -80° C. Both were grown in Marine broth (Difco^{TM}) at 28°C with constant agitation. Densities were determined spectrophotometrically at 550 nm and the calculation was done according to the McFarland standard (BioMerieux, Marcy L'Etoile, France), assuming that an OD550=1.000 corresponds to 1.2×10^{9} cells ml^{-1}.

6.2.2. Gnotobiotic culture of brine shrimp & challenge tests

All experiments were performed with *Artemia franciscana* cysts, originating from Great Salt Lake, Utah, USA (INVE Aquaculture NV, Belgium). Bacteria free cysts and nauplii were obtained via decapsulation according to the procedure described by Sorgeloos *et al.* (1986).

Artemia cysts were hydrated in 9 mL of distilled water for 1 h. Sterile cysts and nauplii were obtained via decapsulation using 0.33 mL NaOH (32%) and 5 mL NaOCl (50%). During the reaction, 0.22 µm filtered aeration was provided. The decapsulation was stopped after about 2 min by adding 5 mL $Na_2S_2O_3$ (10 g l^{-1}). The aeration was then stopped and the decapsulated cysts were washed with filtered (0.22 µm) and autoclaved artificial seawater containing 35 g l^{-1} of Instant Ocean synthetic sea salt (Aquarium Systems, Sarrebourg, France. Procedures were performed under a laminar flow hood to maintain axenic conditions. Equipment was sterilised and autoclaved at 120°C for 20 min. Decapsulated cysts were washed several times over a 100 µm sieve with sterile Instant Ocean (35 g l^{-1}) and transferred to glass tubes. The tubes were placed on a rotor at four cycles per minute and constantly exposed to an incandescent light at 28°C for 18–22 h. Nauplii at instar II stage were collected, counted and transferred to glass bottles with filtered autoclaved sea water (35 g l^{-1}).

Vibrio strain H6 was aseptically inoculated in 30 ml marine broth 2216 (Difco™) and incubated overnight at 25-28°C with constant agitation. Subsequently, 150 µl was transferred and grown to stationary phase in 30 ml marine broth 6 h before challenge. The density was determined spectrophotometrically at an optical density of 550 nm. The bacterial suspension was added to reach a final density of 10^5 cells ml^{-1} for the pathogen and 10^7 cells ml^{-1} for the feed. Each treatment consisted out of 4 replicates. The survival percentage of the brine shrimp larvae was determined 48 h after each respective challenge (Amend, 1981). Verification of the axenity of non-challenged brine shrimp cultures was done by transferring 100 µl of culture medium per bottle to petri dishes containing Marine agar 2216 (n=3). Plates were stored in an incubator for five days at 28°C.

6.2.3. Glucan particles

Insoluble β-1,3/1,6 glucan particles obtained from the yeast *Saccharomyces cerevisiae* (MacroGard) were used. MacroGard is composed out of 60% of β-1,3/1,6 glucans, while the remaining part consists of protein, fat and ash. The particle size distribution was determined by laser diffraction using a Malvern Mastersizer S (Malvern Instruments, Spring Lane South, UK) equipped with a small volume dispersion unit and a 300 RF lens. Distilled water was used as dispersion medium for all the measurements. The volume percentage of particles with a size less than 50 µm was calculated, this being the maximum size that can be ingested by

Artemia (FAO, 1996). Measurements showed that 67.27% of the commercial glucan particles had a diameter lower than 50 µm (Figure 6.1).

The total amount of β-1,3/1,6 glucan particles needed per experiment was calculated as previously described (Marques *et al.*, 2006; Soltanian *et al.*, 2007). The minimum protective amount that was used in both studies corresponded to 3.9 mg l^{-1} for this study. The use of a higher amount of glucans with a range from 10 to 20 mg l^{-1} was also recommended (Soltanian *et al.*, 2007). The β-1,3/1,6 glucan particles were kept in suspension in a tube with filtered and autoclaved sea water (35 g l^{-1}) and stored at 4°C.

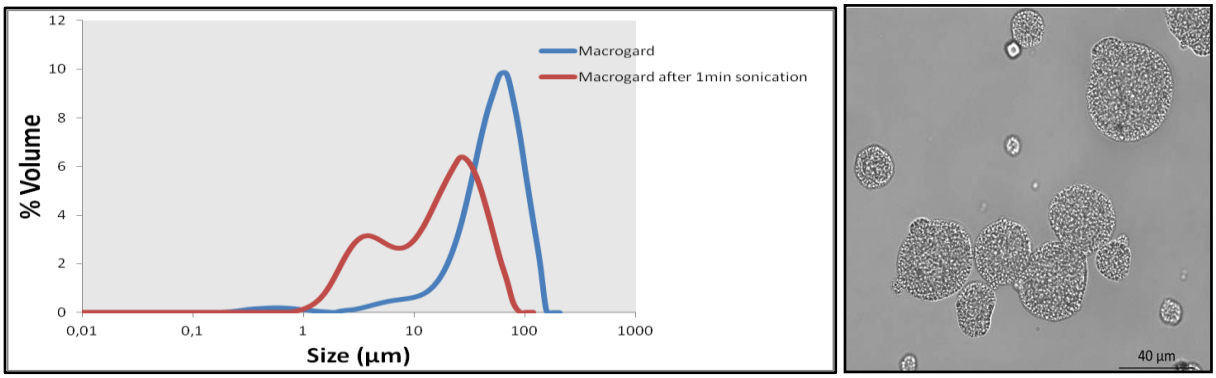

Figure 6.1: Glucan particle volume distribution (%) in relation to the diameter (µm) of MacroGard β-1,3/1,6 glucan particles (left). Microscopic view of the MacroGard β-1,3/1,6 glucan particles (right).

6.2.4. Experimental design

Two experiments were carried out. The first experiment was a dose-response study to determine the appropriate concentration(s) of β-1,3/1,6 glucans providing protection of *Artemia* against H6 challenge. Therefore, groups of 20 nauplii were transferred to sterile 40 ml glass tubes containing 10 ml filtered autoclaved sea water (35 g l^{-1}). Half of the treatments were inoculated with the pathogen H6, the other half were not. All treatments received autoclaved LVS3 as feed, except for one control treatment. β-1,3/1,6 glucan particles were added in three concentrations: a minimum concentration of 3.9 mg l^{-1}, adapted from calculations of Marques *et al.* (2006), and two higher concentrations of 10 and 20 mg l^{-1}. The following groups were maintained as controls: no challenge and no feed (control) and only fed with autoclaved LVS3 but no pathogen. The treatment groups were: addition of β-1,3/1,6 glucan particles in three concentrations (3.9 mg l^{-1}, 10 mg l^{-1}, 20 mg l^{-1}) and addition of β-1,3/1,6 glucan particles together with feed (LVS3 + 3.9 mg l^{-1}, LVS3 + 10 mg l^{-1}, LVS3 + 20

mg l^{-1}). The tubes were placed on a rotor at four cycles per minute and constantly exposed to an incandescent light at 28°C. The survival of *Artemia* was determined 48 h after the challenge by counting the remaining swimming nauplii in each tube. Hence, in this experiment the challenge was performed in the presence of the glucan particles. All manipulations were done under a laminar flow hood in order to maintain axenity. Each treatment was done in quadruplicate for all experiments (n=4).

Table 6.1: Experimental design of the second experiment to examine the effect of a 6 h exposure to 20 mg l^{-1} of β-1,3/1,6 glucans on survival of challenged brine shrimp larvae (n=4). Brine shrimp were fed autoclaved LVS3 in all treatments and were pre-incubated for 6 h, either in FASW or 20 mg β-1,3/1,6 glucans l^{-1} FASW. All larvae were rinsed with FASW after the pre-incubation and were subsequently challenged with *Vibrio* sp. H6 at different time points as indicated.

Group number	Treatment	Preincubation	H6 addition	Sampling time point
1	Control	FASW	-	48h
2	Challenge 0h	FASW	0h	48h
3	Challenge 3h	FASW	3h	51h
4	Challenge 6h	FASW	6h	54h
5	Challenge 12h	FASW	12h	60h
6	Challenge 24h	FASW	24h	72h
7	Glucan control	Glucan	-	48h
8	Glucan + Challenge 0h	Glucan	0h	48h
9	Glucan + Challenge 3h	Glucan	3h	51h
10	Glucan + Challenge 6h	Glucan	6h	54h
11	Glucan + Challenge 12h	Glucan	12h	60h
12	Glucan + Challenge 24h	Glucan	24h	72h

In the second experiment the duration of the protective effect of β-1,3/1,6 glucan against H6 challenge was examined. Axenic *Artemia* nauplii were exposed to the best protective dose of 20 mg l^{-1} β-1,3/1,6 glucans, as observed in the above challenge test, during 6 h.

Subsequently, the nauplii were rinsed with filtered autoclaved sea water (35 g l^{-1}) during 1 minute to remove remaining glucan particles, avoiding any further ingestion of β-1,3/1,6 glucan particles. Twenty nauplii were transferred to sterile glass tubes with 10 ml of FASW (35 g l^{-1}). Dead and autoclaved LVS3 was immediately added as feed at a density of 10^7 cells ml^{-1}. The challenge with H6 (10^5 cells ml^{-1}) was done at different time points: immediately after the washing step and simultaneously with the feeding, 3 h after the washing step and feeding, 6 h, 12 h and 24 h after washing and feeding (Figure 6.2). Controls did not receive any pre-treatment with β-1,3/1,6 glucans, but instead were kept in FASW (35 g l^{-1}) during the same time frame (Table 6.1). The survival of *Artemia* was determined 48h after each respective challenge for each time point (after 48h, 51h, 54h, 60h and 72h respectively). All manipulations were done under a laminar flow hood in order to maintain axenity. Each treatment was done in quadruplicate for all experiments (n=4).

6.2.5. Statistical analysis

Survival data were transformed using arcsin transformation. After wich data was compared a one-way analysis of variances (ANOVA) and weighted least square two-way analysis of variances (ANOVA) with a Tukey's multiple comparisons range test as post hoc analysis. Homogeneity of variances and normality of data were fulfilled. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 21.0 using a significance level of 5%.

6.3. RESULTS

6.3.1. Dose-response study using three concentrations of β-1,3/1,6 glucans

A dose-response study was performed to determine the concentration(s) of β-1,3/1,6 glucans providing optimal protection to pathogenic *Vibrio* sp. H6. Three concentrations of MacroGard were used: 3.9 mg l^{-1}, 10 mg l^{-1} and 20 mg l^{-1} (adapted from Marques *et al.*, 2006). The highest survival was observed for the highest glucan concentration within each group respectively, either with/without feeding or with/without challenge (Table 6.2). The survival increased with rising β-glucan concentration. The addition of LVS3 resulted in a higher survival for all groups. The combination of 20 mg l^{-1} β-1,3/1,6 glucans and LVS3 provided almost complete protection against infection by H6 (86 % survival).

Table 6.2: Survival of gnotobiotic brine shrimp larvae after 48h challenge with *Vibrio* sp. H6 (average % ± standard deviation of four replicates). "Control" refers to animals that were not challenged or exposed to glucans, but otherwise treated in the same way as the other larvae. Data with different superscripts are significantly different in a one-way ANOVA analysis ($P < 0.05$).

Treatment	Unchallenged (survival % ± SD)	Challenged with H6 (survival % ± SD)
Control	$79 \pm 3^{d,e}$	5 ± 4^{a}
Glucan 3.9 mg l^{-1}	$93 \pm 3^{f,g}$	36 ± 3^{b}
Glucan 10 mg l^{-1}	$89 \pm 3^{e,f}$	43 ± 12^{b}
Glucan 20 mg l^{-1}	$93 \pm 3^{f,g}$	$69 \pm 5^{c,d}$
LVS3	81 ± 3^{e}	34 ± 7^{b}
LVS3 + glucan 3.9 mg l^{-1}	98 ± 3^{g}	44 ± 5^{b}
LVS3 + glucan 10 mg l^{-1}	98 ± 3^{g}	68 ± 7^{c}
LVS3 + glucan 20 mg l^{-1}	99 ± 3^{g}	$86 \pm 3^{e,f}$

To check for interactions, two-way ANOVA analyses were performed, namely one for all treatments in which LVS3 was added as feed and one for all treatments without LVS3. The factors in the two-way ANOVA were glucan concentration and challenge. This allows to check for the effect of the β-1,3/1,6 glucans in combination with LVS3 and without the background noise of this feed. There was a significant interactive effect ($P < 0.001$) for the glucan concentration and the challenge, indicating that the glucans provided protection against the pathogen (outcome of the statistical analysis not shown).

6.3.2. Duration of the protective effect of β-1,3/1,6 glucans

In a second experiment it was determined for how long the glucan treatment offered protection against a challenge with *Vibrio* sp. H6. To this end, axenic *Artemia* nauplii were either or not exposed to 20 mg/l β-1,3/1,6 glucans, after which all larvae were rinsed. Subsequently different groups of larvae were challenged with *Vibrio* sp. H6 at different time points (0, 3, 6, 12 and 24 h after rinsing, respectively). All groups were fed with LVS3 and survival was counted 48h after challenge (i.e. 48, 51, 54, 60 and 72h after rinsing,

respectively). Overall, the addition of β-glucans enhanced survival in the challenged and control groups as compared to untreated nauplii (Table 6.3).

Table 6.3: Survival of gnotobiotic brine shrimp larvae 48h after challenge with pathogenic *Vibrio* sp. H6 (average ± standard deviation of four replicates). Larvae were either or not pre-exposed to 20 mg l^{-1} β-1,3/1,6 glucans for 6 h, after which all larvae were rinsed. Larvae were subsequently challenged with *Vibrio* sp. H6 at different time points. "Control" refers to animals that were not challenged or exposed to glucans, but otherwise treated in the same way as the other larvae. Data within individual rows with different superscripts are significantly different in a one-way ANOVA analysis ($P < 0.05$).

Treatment	**Time of challenge (total rearing time after rinsing)**				
	0h (48h)	**3 h (51h)**	**6 h (54h)**	**12 h (60h)**	**24 h (72h)**
Control	69 ± 5^{b}	64 ± 4^{b}	55 ± 4^{b}	40 ± 4^{b}	2 ± 3^{b}
Glucan	90 ± 4^{a}	86 ± 4^{a}	75 ± 3^{a}	70 ± 5^{a}	65 ± 5^{a}
Challenge	19 ± 5^{d}	15 ± 5^{d}	9 ± 3^{d}	1 ± 4^{c}	0 ± 3^{b}
Glucan + challenge	50 ± 4^{c}	36 ± 4^{c}	30 ± 3^{c}	9 ± 5^{c}	3 ± 4^{b}

In all groups, survival gradually declined. Survival was still remarkably high 72h after rinsing in the unchallenged glucan-treated group, whereas in the unchallenged control group, almost all nauplii were dead at this time point. For the challenged groups there is a significant effect of pre-treatment with β-1,3/1,6 glucans on survival when challenged up to 6 h after rinsing. Overall, the β-1,3/1,6 glucans did have a significant effect on the survival of the *Artemia* nauplii and this effect is visible for at least 6 h after which it decreases.

6.4. DISCUSSION

The brine shrimp *Artemia franciscana*, as all invertebrates, depends only on its innate immune mechanisms and does not have adaptive immune response or immunological memory (Cerenius & Söderhäll, 1995). However, studies have shown the presence of some form of specific immunity in invertebrates by priming activities (Kurtz & Franz, 2003). Conclusive data about priming were obtained by Little *et al*. (2003). Little and colleagues found that a subsequent exposure with the pathogen *Pasteuria ramosa* increased the survival and reproductive fecundity of the water flea *Daphnia pulex*. A study with larvae of the mealworm

beetle, *Tenebrio molitor*, showed that an injection with lipopolysaccharides (LPS) prior to challenge increased survival after a later exposure to the fungus *Metarhizium anisopliae* (Moret & Siva-Jothy, 2003). Studies on decapods have shown that the primed status might last between 3 and 72 h (Smith *et al*. 2003). Research on bumble bees showed that immune priming can extend for days and weeks, sometimes even across the different life stages (Schmid-Hempel, 2005), which suggests changes in the epigenetic landscape. A study on *Drosophila* showed that a sub lethal dose of *Streptococcus pneumonia* protected the animal against a second lethal challenge with the same pathogen. The protective effect persisted during the whole lifespan of the fly, but this primed response was not induced by different microbial challenges (Pham *et al*., 2007).

A study from Soltanian *et al*. (2007) showed that baker's yeast (*Saccharomyces cerevisiae*) mnn9 improved *Artemia* survival against a *Vibrio campbellii* infection. Mnn9 is a mutant which lacks cell wall-bond mannoproteins and contains more glucan and chitin. Previous studies also showed that arbitrary use of these immunostimulants without prior knowledge of the optimum dose and frequency may cause immunosuppression resulting in a collapse of the immune system (Sajeevan *et al*., 2009). Several studies have reported that β-glucans enhance resistance of crustaceans against various diseases, including bacterial and viral diseases (Soltanian, 2007; Su *et al*., 1995; Liao *et al*., 1996; Chang *et al*., 2000; Marques *et al*., 2006). In spite of its efficiency, immunostimulation with β- glucans is not only dependent on the quantity, but also on the quality of the commercial product, such as molecular weight, three dimensional structures and branching frequency (Soltanian *et al*., 2007). As a consequence, a commercial immunostimulation product often requires frequent validation (Alabi *et al*., 2000). A particular problem is to determine the effective dose and time period of the β-1,3/1,6 glucan administration (Sung *et al*., 1996; Alabi *et al*., 1999; Vici *et al*., 2000). Here, we performed a dose-response study to determine the appropriate concentration(s) of β-1,3/1,6 glucans offered to *Artemia* against the virulent pathogen *Vibrio* sp. H6 (Vanmaele *et al*., 2015). The results indicated that β-1,3/1,6 glucan-exposed *Artemia* nauplii were significantly better protected against a challenge when compared to control groups. The highest survival was observed in the group where the combination of LVS3 and 20 mg l^{-1} of β-1,3/1,6 glucans were added to the culture system. This could partially be related to a feeding effect as MacroGard is not a pure glucan. During these experiments the pathogen was added at a concentration of 10^5 cells ml^{-1} while the feed was added at a concentration of

10^7 cells ml^{-1}. This was done based on a study by Defoirdt & Sorgeloos (2012) who showed that final concentrations of the pathogen will increase in the glass tubes. Therefore concentrations of the pathogens were lowered, observed survival remained low in the control group that received challenge.

In the second part of this study the duration of the protective effect of β-1,3/1,6 glucans was examined. A protective effect was observed in the challenged groups treated with glucans during 6 h and challenged with *Vibrio* sp. H6, until 6 h post glucan removal. When the pathogen was added 12 h after the priming period, this protection was no longer present. This implies that an exposure period of 6 h with β-1,3/1,6 glucans (20 mg l^{-1}) protected the nauplii for at least 6 h as revealed by a challenge with H6. In this experiment 20 mg l^{-1} of β-1,3/1,6 glucan particles did not harm the nauplii. Indeed, in the unchallenged groups the survival gradually declined until almost all nauplii were dead at the last sampling point, while in the β-1,3/1,6 glucans group survival was still around 65%. This indicates that the used concentration is not detrimental for the host, but rather provides a nutritional benefit for the animals.

A previous study (Campbell *et al*,. 1993) showed that in the presence of food, *Artemia* had a constant uptake level for 10-30min. When animals were transferred to sea water this level dropped, indicating that the gut was emptied due to the stress of washing (Campbell *et al*. 1993). Since a similar procedure was performed in this study compared to Campbell's study, we could hypothise that the glucans would not remain in the gut after the washing step in our experiment as well, although this was not experimentally confirmed.

The results showed that β-1,3/1,6 glucans primed the *Artemia* immune system resulting in a significantly improved survival upon a pathogen exposure. This protection lasted for 6 h. The results also suggest that β-1,3/1,6 glucans activate the innate immune system of invertebrates, such as *Artemia,* to provide protection against a pathogen (Soltanian, 2007; Marques, 2006). Further research is however recommended to clarify the mechanisms involved in this immune response.

6.5. CONCLUSION

In this study, we characterised the effect of β-1,3/1,6-glucans on the survival of gnotobiotic *Artemia franciscana* nauplii upon challenge with a virulent *Vibrio* strain H6. Our results showed a significant improved survival of *Artemia* nauplii after exposure to the β-1,3/1,6-glucans (MacroGard) at a concentration of 20 mg l^{-1}. This protection lasted for 6 h. Further research is warranted to elucidate the mechanisms involved in the protection of *Artemia* by β-1,3/1,6 glucans against pathogens.

CHAPTER 7

EFFECT OF BETA-GLUCANS ON THE EXPRESSION OF IMMUNE-RELATED GENES IN GNOTOBIOTIC *ARTEMIA FRANCISCANA*

In collaboration with Romi Novriadi, D. Mundanda Muthappa Sonydechu, dr. Bert Devriendt, Prof. dr Eric Cox, Prof. dr. ir Peter Bossier

CHAPTER 7:

Effect of β-glucans on the expression of immune-related genes in gnotobiotic *Artemia franciscana*

ABSTRACT

Immunostimulants (e.g. Hsp70 and β-glucans) are known to activate the immune system of crustaceans and tend to protect against a wide range of pathogens. The pathways, genes and modes of action involved though still remain largely unknown. In this study, we determined the impact of immunostimulants on the expression of genes encoding proteins involved in the innate immune response, including the Down syndrome cell adhesion molecule (Dscam), Masquarade like protein (Masq), Lipopolysaccharide and β-1,3-glucan binding protein (LGBP), Peroxinectine (Pero), Transglutaminase (Tgase), Prophenoloxidase (ProPO), extracellular superoxide dismutase (eSOD) and Heat Shock Protein 70 (Hsp70). The immune response towards β-1,3/1,6-glucans was investigated in a gnotobiotic model, using brine shrimp (*Artemia franciscana*) in combination with the virulent strain *Vibrio* sp. H6 in a challenge test. Commercial β-glucans from a specially selected strain of *Saccharomyces cerevisiae* (MacroGard) were used. The impact of β-glucan on gene expression was evaluated at different time points after exposure using real-time qPCR. Results showed significant up-regulations for *masq* (a 563-fold increase) and down-regulations for *esod* and *pero* at 12 h after a β-glucans exposure. The β-1,3/1,6-glucans exposure had a significant effect on the expression of *pero*, *masq* and e*sod* levels. In conclusion, β-glucan particles did stimulate the innate immune system in brine shrimp, thereby providing protection to the animals upon exposure to the *Vibrio* sp. H6.

7.1. INTRODUCTION

As the prophylactic use of antibiotics is slowly being phased out, immunostimulants and probiotics have been used as approache to control diseases in aquaculture with β-glucans being amongst the most intensively used compounds (FAO, 2012; Marques *et al*., 2006; Bachère 2003; Smith *et al*., 2003). They are glucose polymers that are found in fungi, plants, including seaweeds, and some bacteria. Exposure to β-glucans activates immune responses and improves survival during a subsequent exposure to pathogenic micro-organisms (Jiravanichpaisal *et al*., 2006; Kurtz & Franz, 2003; Hauton & Smith, 2007).

In invertebrates, β-glucans trigger the coagulation of the hemolymph, production of antimicrobial peptides and activate the melanisation cascade that converts prophenoloxidase (ProPO) into catalytically active phenoloxidase (PO) (Soltanian *et al*., 2009; Cerenius *et al*., 2010; Sivakam *et al*., 2012; Liu *et al*., 2006). The innate immune defence of crustaceans consists of a number of factors, of which transglutaminase and prophenoloxidase are the most intensively studied ones. Tgase and proPO are known indicators of an activated immune system and their presence accelerates the clearance of the infection. Transglutaminases are involved in the clotting system, and plays a role in the molting process to prevent blood loss from wounds and to obstruct pathogens from invading the host via wounds (Liu *et al*., 2007). Transglutaminases (Tgase) are produced by the hemocytes as a response to tissue damage (Hall *et al*., 1999; Wang *et al*., 2001). The prophenoloxidase (ProPO) activating system is involved in melanisation. It is one of the major immune defence mechanisms against several pathogens for invertebrate animals (Cerenius & Söderhäll, 2004). Prophenoloxidase is turned into phenoloxidase by a cascade of serine proteinases, and this reaction is triggered by pathogen-related compounds (pathogen-associated molecular patterns) such as β-glucans, lipopolysaccharides and peptidoglycans.

Heat shock proteins (such as Hsp70) are involved in the folding and unfolding of other proteins (intracellular) and are induced when the host is exposed to high temperatures, salinity variation or other stress factors (Baruah, 2012; Sung & MacRae, 2011; Zhou *et al*., 2010). Extracellularly, they induce immune responses through Toll-like receptors, whereby the accumulation of Hsp70 is related to a higher tolerance for disease (Hu *et al*., 2014). In

Artemia franciscana studies have mostly been focused on Hsp70 (Baruah *et al*., 2010 & 2012).

Extracellular Superoxide Dismutase (eSOD) plays a major role as an antioxidant defence mechanism. This enzyme turns superoxide (O_2^-) into oxygen (O_2) and hydrogen peroxide (H_2O_2), thereby initiating the removal of reactive oxygen species from the host's body (Holmblad & Söderhall, 1999). Down syndrome cell adhesion molecules (Dscam) are crustacean receptors that are involved in neural development and they play a role in the detection of pathogen-associated molecular patterns (Hauton, 2012; Watthanasurorot *et al*., 2011). They play an essential role in the innate immune system of crustaceans by producing a wide variety of isoforms with each specific binding capability for a certain immune challenge or infection (Rowley & Pope, 2012; Brites *et al*., 2008). The Masquarade-like protein has cell adhesion properties and can bind to the cell wall of Gram-negative bacteria and yeasts, after which the Masq-like protein is processed by a proteolytic enzyme and functions as an innate immune protein (Lee & Söderhall, 2001; Cerenius & Söderhall, 2004). Lipopolysaccharide and β-1,3-glucan binding protein (LGBP) is a pattern recognition receptor for lipopolysaccharides and β-1,3/1,6-glucans , which are present in Gram-negative bacteria and fungi, respectively (Yang *et al*., 2010; Yu *et al*., 2001). Peroxinectins are proteins involved in cell adhesion, peroxidase activity and encapsulation of pathogens (Johansson *et al.,* 1988; Thörnqvis *et al*., 1994). Peroxinectin is associated with the activation of the ProPO system. The recognition of lipopolysaccharides and β-glucans triggers the expression of peroxinectin (Johansson *et al*., 1999), which in turn activates the prophenoloxidase cascade, resulting in phagocytosis or encapsulation of pathogens (Hauton, 2012).

Artemia franciscana is frequently being used as a gnotobiotic model system to study host-microbial interactions and immune responses (Vanhaecke *et al*., 1981; Overton & Bland, 1981; Criado-Fornelio *et al*., 1989; Verschuere *et al*., 1999 & 2000; Marques *et al*., 2004 & 2005; Soltanian, 2007; Baruah, 2012). They have unique characteristics which make them ideal candidates for being a gnotobiotic crustacean model system. A good model system though, also allows for genetic manipulation, which is not yet the case for *Artemia franciscana*, yet the future looks promising with the recent estimation of the genome (De Vos *et al*., 2013) and the current annotation of the genome (Ghent University non-disclosed information at the Lab Aquaculture & *Artemia* Reference Center). Until now, however,

information on the mode of action of β-glucans on the immune response of brine shrimp remains scarce, especially with respect to the gene expression level. In this study, we aimed at determining the impact of β-glucans on the expression of eight genes (some of them merely identified*, based on homology with *Pacifastacus* peptide, see Appendix) that are involved in the innate immune response: Down syndrome cell adhesion molecule (*dscam**), Masquarade like protein (*masq**), Lipopolysaccharide and β-1, 3-glucan binding protein (*lgbp**), Peroxinectine (*pero**), Transglutaminase (*tgase*), Prophenoloxidase (*proPO*), extracellular superoxide dismutase (e*SOD**) and Heat Shock Protein 70 (*hsp70*).

7.2. MATERIALS & METHODS

7.2.1. Bacterial strains and culture conditions

The isolate H6, belonging to the *V. harveyi* clade (Vanmaele *et al*., 2015), was used as a pathogenic strain in the challenge test. The autoclaved *Aeromonas sp.* strain LVS3 was used as a feed source for the *Artemia franciscana* larvae (Verschuere *et al*., 1999). The bacterial isolates H6 and LVS3 were grown in marine broth (Difco^{TM}) at 28°C with constant agitation. Densities were determined spectrophotometrically at 550 nm and the calculation was done according to the McFarland standard (BioMerieux, Marcy L'Etoile, France), assuming that an OD550=1.000 corresponds to 1.2×10^9 cells ml^{-1}.

7.2.2. Gnotobiotic culture of brine shrimp & challenge tests

All experiments were performed with *Artemia franciscana* cysts, originating from Great Salt Lake, Utah, USA (INVE Aquaculture NV, Belgium). Bacteria-free cysts and nauplii were obtained via decapsulation according to the procedure described by Sorgeloos *et al*. (1986). *Artemia* cysts were hydrated in 9 ml of distilled water for 1 h. Sterile cysts and nauplii were obtained via decapsulation using 0.33 ml NaOH (32%) and 5 ml NaOCl (50%). During the reaction, 0.22 μm filtered aeration was provided. The decapsulation was stopped after about 2 min by adding 5 ml $Na_2S_2O_3$ (10 g l^{-1}). The aeration was then stopped and the decapsulated cysts were washed with filtered (0.22 μm) and autoclaved artificial seawater containing 35 g l^{-1} of Instant Ocean synthetic sea salt (Aquarium Systems, Sarrebourg, France. Procedures were performed under a laminar flow hood to maintain axenic conditions. Equipment was sterilised and autoclaved at 120°C for 20 min. Decapsulated cysts were washed several times over a

100 µm sieve with sterile Instant Ocean (35 g l^{-1}) and transferred to glass tubes. The tubes were placed on a rotor at four cycles per minute and constantly exposed to an incandescent light at 28°C for 18 – 22 h. Nauplii at instar II stage were collected, counted and transferred to glass bottles with filtered autoclaved sea water (35 g l^{-1}). The bacterial suspension was added to reach a final density of 10^5 cells ml^{-1} for the pathogen and 10^7 cells ml^{-1} for the feed. Each treatment was carried out in duplicate.

Verification of the axenity of non-challenged brine shrimp cultures was done by transferring 100 µl of each culture medium to petri dishes containing Marine agar 2216 (n=3). Plates were stored in an incubator for five days at 28°C.

7.2.3. Glucan particles

Pure insoluble β-1,3/1,6 glucan particles obtained from the yeast *Saccharomyces cerevisiae* (MacroGard) were used. See Chapter 6 (6.2.3) for preparation and administration of the particles during the experiment.

7.2.4. Experimental design

The effects of a pre-treatment of the *Artemia* nauplii with β-1,3/1,6 glucans in combination with a challenge on the expression of eight immune-related genes in *Artemia* were investigated (Table 7.1). *Artemia* nauplii were exposed to the β-1,3/1,6 glucan particles at a concentration of 20 mg l^{-1} for 6 h, the control group did not receive any pre-treatment. The nauplii were rinsed repeatedly to remove the glucan particles. After which both the feed, dead LVS3 particles at a concentration of 10^7 cells ml^{-1}, and the pathogen H6 (10^5 cells ml^{-1}) were added simultaneously. Samples containing 0.1g of live nauplii were harvested for all treatments at the different sampling points: 0h, 3 h, 6 h and 12 h after *Vibrio* exposure and feeding. Two biological replicates per treatment were sampled (n=2). Nauplii were rinsed in cold distilled water and immediately frozen in liquid nitrogen and stored at -80°C.

7.2.5. Primer design

The primers for *hsp70, proPO* and *tgase* were previously described by Niu *et al*. (2014). For the other five genes (e*SOD, masq, pero, dscam* and *lgbp*) primers were designed (Ghent University non-disclosed information at the Lab Aquaculture & *Artemia* Reference Center, Dechamma *et al.,* unpublished). Sequences from crayfish *Pacifastacus* corresponding to

these immune genes were retrieved from the National Center for Biotechnology Information (NCBI) and blasted against the *Artemia franciscana* genome database (Internal UGent information). Blast hits showing maximum similarity were chosen to design the primers (See Appendix). Amplification products were resequenced to confirm the genes identified by BLAST. Although the gene products show very high similarity with the corresponding *Pacifastacus* genes and/or other invertebrate genes, evidence for their functionality awaits further research. Primers were designed using the online software Primer Express® Software v3.0.1 (Primer 3, IDT) and obtained from Eurogentec (Seraing, Belgium).

Table 7.1: Experimental design to examine the effect of a 6 h exposure to 20 mg l^{-1} of β-1,3/1,6 glucans on gene expression of challenged brine shrimp larvae (n=2). Brine shrimp were fed autoclaved LVS3 in all treatments and were pre-incubated for 6 h, either in FASW or 20 µg β-1,3/1,6 glucans per ml FASW. All larvae were rinsed with filtered and autoclaved sea water (FASW) after the pre-incubation and were subsequently challenged with *Vibrio* sp. H6. Sampling was done at different time points, as indicated.

Group number	Treatment	Preincubation	H6 addition	Sampling time point
1	Control	FASW	-	0h
2	Challenge 0h	FASW	0h	0h
3	Challenge 3h	FASW	0h	3h
4	Challenge 6h	FASW	0h	6h
5	Challenge 12h	FASW	0h	12h
6	Glucan control	Glucan	-	0h
7	Glucan + Challenge 0h	Glucan	0h	0h
8	Glucan + Challenge 3h	Glucan	0h	3h
9	Glucan + Challenge 6h	Glucan	0h	6h
10	Glucan + Challenge 12h	Glucan	0h	12h

7.2.6. Gene expression

Total RNA was extracted from the *Artemia* nauplii using the SV Total RNA Isolation System (Promega) kit according to the manufacturer's instructions (which includes DNAse

treatment), after which the concentration and purity of the RNA was quantified spectrophotometrically (NanoDrop Technologies, Wilmington, DE, USA). First strand cDNA was synthesised from 1 µg total RNA using RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas Gmbh, Germany) according to the manufacturer's instructions. The gene expression in *Artemia* was analysed by qPCR using specific primers (Table 7.2), all of them showing a similar efficiency.

Table 7.2: Amplicon length (bp) of the eight primers used during the experiment.

			Amplicon length				
tgase	*proPO*	*hsp70*	*dscam*	*masq*	*pero*	*lgbp*	*esod*
149bp	98bp	182bp	111bp	122bp	111bp	123bp	132bp

The qPCR amplifications were carried out in a total volume of 25 µl, containing 12.5 µl of Maxima SYBR Green qPCR Master mix (Fermentas, Cambridgeshire) primer (concentrations see Table 7.2) and 2 µl cDNA template. The qPCR was performed in a StepOne qRT-PCR instrument (Applied Biosystems) using a four-step amplification protocol: initial denaturation (2 min at 50°C and 10 min at 95^{o}C); 40 cycles of amplification and quantification (15 s at 95^{o}C, 1 min at annealing temperature (Table 7.3)); melting curve (15 s at 95°C, 1 min at 60°C and 15 s at 95°C and a continuous fluorescence measurement) and cooling (4^{o}C). The β-actin gene was used as an internal reference gene. Relative quantification of target gene transcripts with a chosen reference gene transcript was done following the ΔΔCT method (Livak & Schmittgen, 2001). Four technical replicates for each to the two biological replicates were used during the calculations. The expression of the target genes was normalised to the reference gene by calculating ΔCT (= CTtarget - CTactin) and expressed relative to a calibrator strain by calculating ΔΔCT (= ΔCT - ΔCTcalibrator). The calibrator was the control treatment at the T0 time point. The relative expression was then calculated as $2^{-\Delta\Delta Ct}$.

Table 7.3: Primers used for real-time quantitative PCR amplification.

Gene	Efficiency	Concentration	Annealing Temperature	Strand	Sequences of the forward and reverse primers (5' – 3')
β-actin	2.0	0.20µM	60°C	F	5'-AGCGGTTGCCATTTCTTGTT -3'
				R	5'-GGTCGTGACTTGACGGACTATCT-3'
proPO	2.2	0.20µM	60°C	F	5'-TCTGCAAGGAGGATTTAAGGA -3'
				R	5'-TGACTGACAAAGGAGATGGGAC -3'
hsp70	2.1	0.20µM	60°C	F	5'-CGATAAAGGCCGTCTCTCCA -3'
				R	5'-CAGCTTCAGGTAACTTGTCCTTG -3'
tgase	2.2	0.20µM	60°C	F	5'-TCTCTCCGTGTCTCTCCAAAAG -3'
				R	5'-CCCCACAAGAAGCATCTGAAG -3'
esod	1.9	0.25µM	60°C	F	5'-TGGTGGTCCAGATGATAC-3'
				R	5'-TTCTTTCTCCGCATAGGG-3'
pero	2.0	0.25µM	60°C	F	5'- GAGCTACCGATGAAGATCCAG-3'
				R	5'- CGTTTCCTGAACAGCGAATAAA -3'
dscam	1.9	0.25µM	51°C	F	5'- CATAGTCCAAGAGTGAATG -3'
				R	5'- GCCACATATTCAGTTAGAA -3'
masq	1.9	0.20µM	58°C	F	5'-CTCCTACAGTTCGCTTCT-3'
				R	5'-GTCTGCCGTCAATCAAAG-3'
lgbp	2.0	0.20µM	50°C	F	5'- GTGGACTGATGCTGAATG -3'
				R	5'- CATAGCCAGGGACAGAA -3'

7.2.7. Statistical analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 21.0 using a significance level of 5%. Due to practical difficulties only two biological replicates were sampled. Homogeneity of variances and normality of data were not fulfilled for an ANOVA analysis. Therefore, differences in mRNA expression between control and treatments were analysed using independent samples t-tests after log-transforming the expression values. All treatments were compared to the blank and compared to each of the other treatments individually for each time point respectively. Per time point, a matrix was obtained with an overview of all the results from the independent samples t-tests. The outcome was condensed and presented in the graphs.

7.3. RESULTS

The objective of this study was to determine the effect of β-glucans, the effect of the *Vibrio* challenge and how pre-exposure to β-glucans affects *Vibrio*-induced gene expression patterns on innate immune gene expression in brine shrimp. In order to determine these outcomes the mRNA profiles of eight immune-related genes were monitored and calculated relative to those of a housekeeping gene. The housekeeping gene, β-actin, was stable over all samples and sampling points of the experiment, no abnormalities were observed.

For the Down syndrome cell adhesion molecule no statistical significant differences were observed in the expression levels between treatments at each respective time point (Figure 7.1). Nonetheless patterns in mRNA expression levels can be observed. Expression levels for *dscam* show a similar expression pattern among treatments at each respective time point, except 12 h after the washing step. At 3 h and 6 h, with respect to the blank, an increase can be observed in expression levels with lowest values observed for the blank, followed by the β-glucan treatment, *Vibrio* sp. H6 and β-glucan+ *Vibrio* sp. H6. At 12 h, the β-1, 3-glucans induced a 10- and 6-fold increase in expression levels for treatments β-glucans and β-glucans + *Vibrio* sp. H6, respectively. At all time points higher expression levels can be observed for both groups that received the β-1, 3-glucan pre-treatment compared to their non-treated counterparts, independent of an exposure to the *Vibrio sp.* H6.

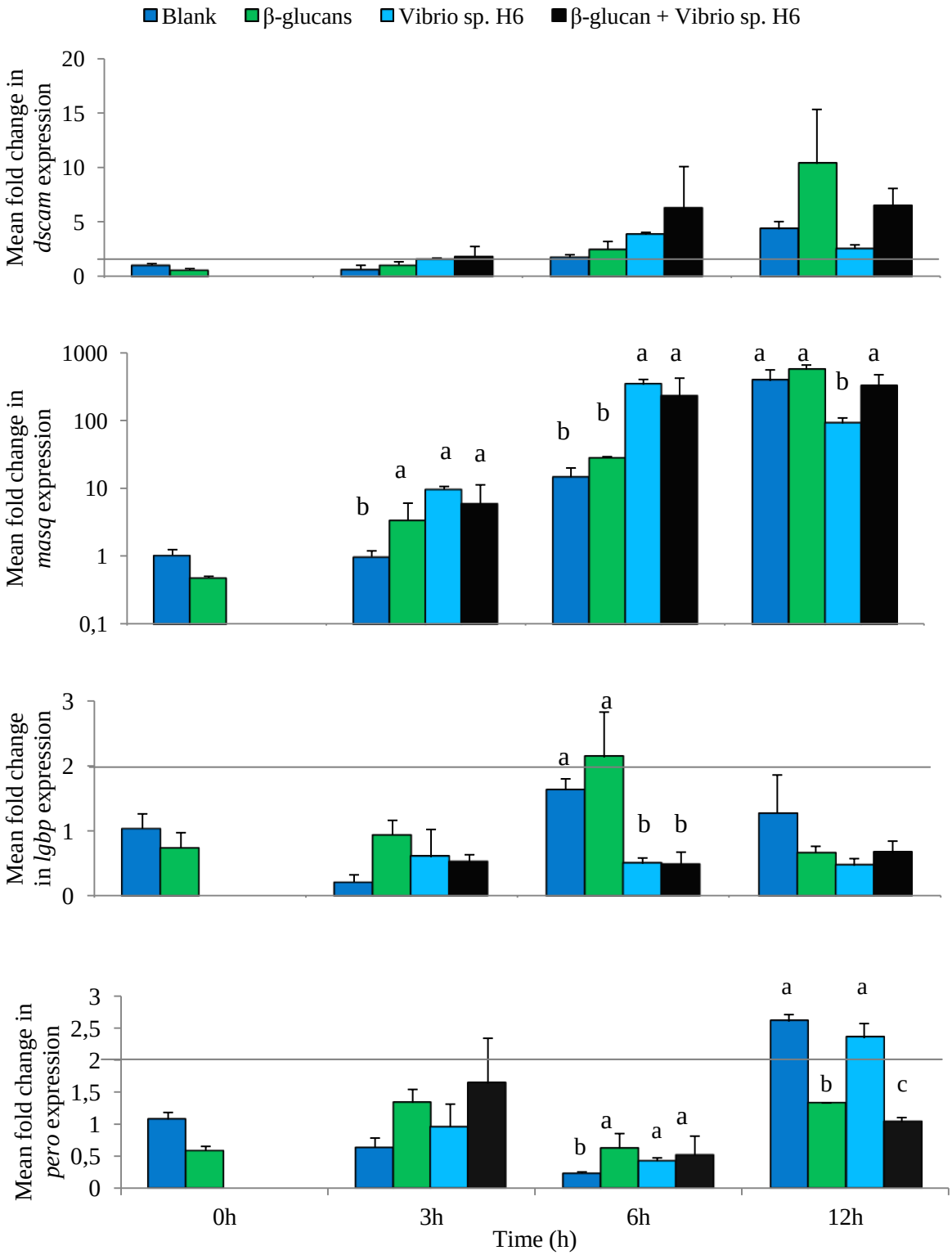

Figure 7.1: Relative mean fold change in gene expression of *dscam*, *masq*, *lgbp* and *pero*. Blank: control; β-glucans: pre-treatment with β-1,3/1,6 glucans during 6 h prior to the start of the experiment; *Vibrio* sp. H6: challenge with *Vibrio* sp. H6; β-glucans + *Vibrio* sp. H6: pre-treatment with β-1,3/1,6 glucans during 6 h followed by challenge with *Vibrio* sp. H6. The expression was calculated relative to the β-actin gene; expression in untreated brine shrimp at the start of the experiment was set at 1 and the other datapoints were normalised accordingly. Error bars indicate the standard error. For each time point specifically, different letters indicate significant differences ($P < 0.05$).

For *masq*, significant differences in expression levels were observed at several time points (Figure 7.1). At 3 h there was a significant difference between the control and all the other groups, indicating that the glucans and/or the *Vibrio* are increasing the gene expression. At 6 h there was significant higher expression levels in both challenged groups (*Vibrio* sp. H6) than in the blank (control) and than in the glucan group, pointing to an important impact of *Vibrio* at that time point. Yet, at time point 12 h expression patterns change. Very high expression levels were observed after 12 h for all groups. In the glucan treatment and the glucan/H6 treatment expression levels are statistically equal to the blank. A single exposure to *Vibrio* significantly lowers the expression level relative to the blank. For lipopolysaccharide and β-1, 3-glucan binding protein statistical significant changes were observed in expression at 6 h (Figure 7.1) with a significantly lower expression of *lgbp* in the challenged groups than in the unchallenged groups. Twelve hours after the washing step, no significant effect of the β-glucan treatment could be observed for the *lgbp* gene.

The expression pattern of *pero* showed significant changes at 6 h and 12 h. At 3 h a slight but non-significant increase due to the β-1,3/1,6 glucan treatment was seen. At time point 6 h a significant up-regulation occured in all treatments, compared to the control group. However, 12 h after the washing step a significant down-regulation, was observed for both groups that received the β-1,3/1,6 glucan treatment (Figure 7.1), with lowest expression levels for both the joint β-1,3/1,6 glucan and *Vibrio sp.* H6 exposure.

For *tgase* no statistically significant changes in expression levels per time point were observed (Figure 7.2). At the 3 h time point, expression levels of *proPO* tend to be high in all treatments (Figure 7.2), with a gradual decrease from that point onwards. β-1,3/1,6 glucan exposure in absence of the pathogen significantly delayed the decrease at 6 h (2-fold higher expression), whereas challenge completely abolished the effect of the β-1,3/1,6 glucans.

The extracellular superoxide dismutase gene (esod) showed very different expression patterns between the different time points (Figure 7.2). In the control group the expression remains stable until 6 h after rinsing with FASW, whereafter it dropped at 12 h. Preincubation with glucans significantly decreased expression (at 0 and 3 h), whereafter a significant (3-fold) increase occurred at 6 h.

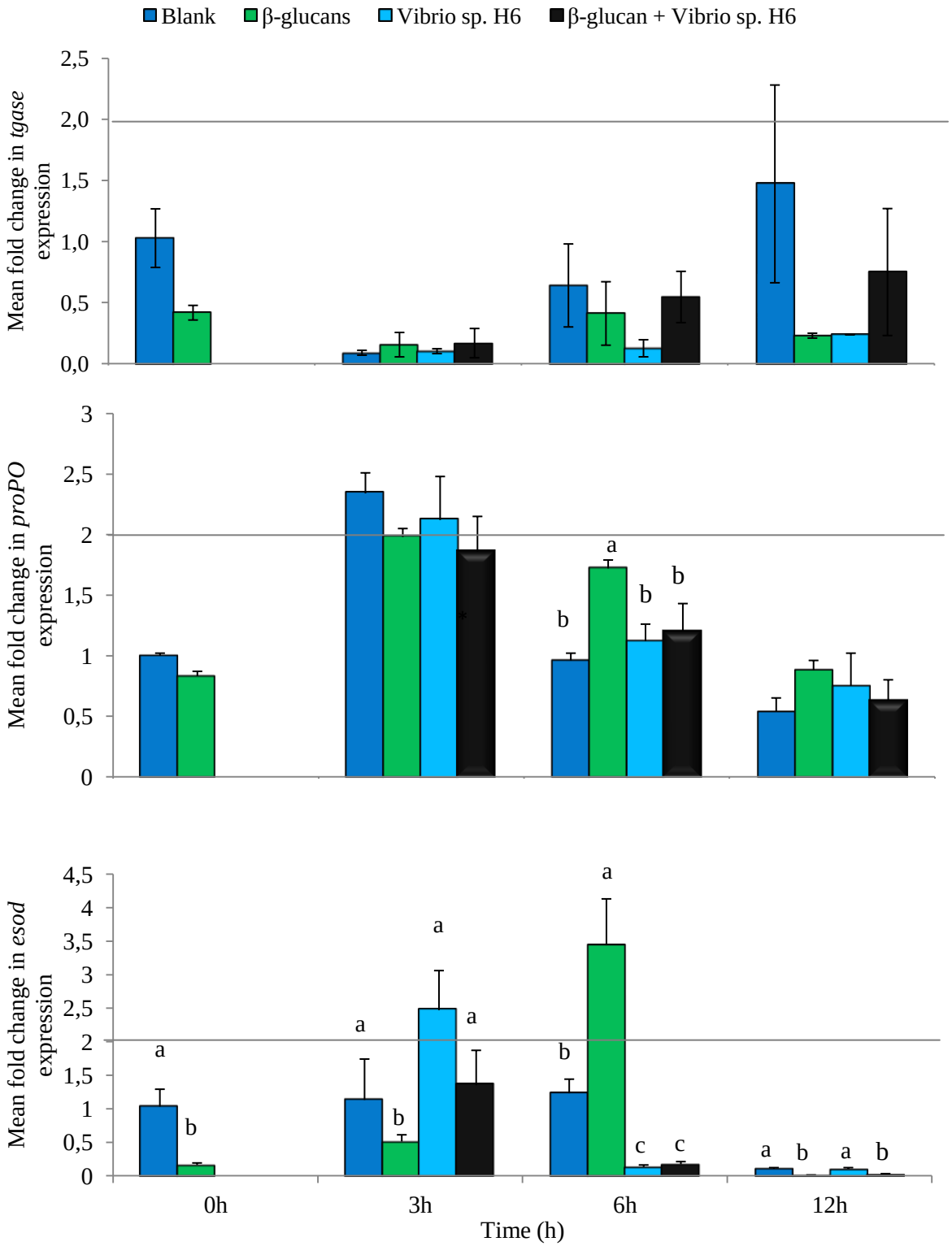

Figure 7.2: Relative mean fold change in gene expression of *tgase*, *proPO* and *esod*. Blank: control; β-glucans: pre-treatment with β-1,3/1,6 glucans during 6 h prior to the start of the experiment; *Vibrio* sp. H6: challenge with *Vibrio* sp. H6; β-glucans + *Vibrio* sp. H6: pre-treatment with β-1,3/1,6 glucans during 6 h followed by challenge with *Vibrio* sp. H6. The expression was calculated relative to the β-actin gene; expression in untreated brine shrimp at the start of the experiment was set at 1 and the other datapoints were normalised accordingly. Error bars indicate the standard error. For each time point specifically, different letters indicate significant differences ($P < 0.05$).

However, in the presence of the challenge infection neither a decrease at 3h, nor an increase at 6 h was observed. At 6h the *Vibrio* exposure, significantly decreased the expression to values that were close to zero. At the time point of 12 h, expression levels were very low in for all treatments, Nevertheless, a significant down-regulation of expression could be observed as a result of the β-1,3/1,6 glucan treatment.

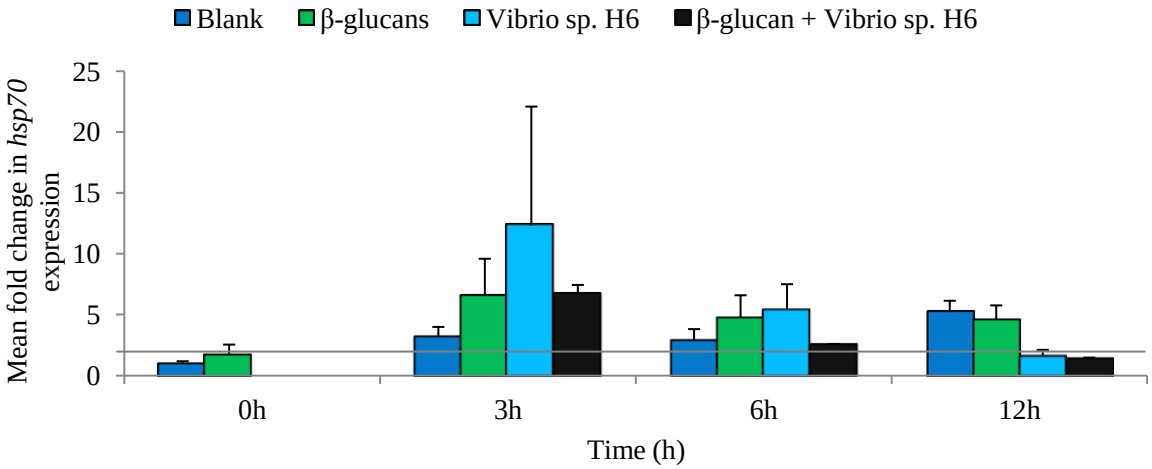

Figure 7.3: Relative mean fold change in gene expression of *hsp70*. Blank: control; β-glucans: pre-treatment with β-1,3/1,6 glucans during 6 h prior to the start of the experiment; *Vibrio* sp. H6: challenge with *Vibrio* sp. H6; β-glucans + *Vibrio* sp. H6: pre-treatment with β-1,3/1,6 glucans during 6 h followed by challenge with *Vibrio* sp. H6. The expression was calculated relative to the β-actin gene; expression in untreated brine shrimp at the start of the experiment was set at 1 and the other datapoints were normalised accordingly. Error bars indicate the standard error. For each time point specifically, different letters indicate significant differences ($P < 0.05$).

No significant changes in expression were observed for *hsp70* at any time point. Both the β-glucan treatment and *Vibrio* challenge tended to up-regulate *hps70* expression at time point 3 h and 6 h, but not significantly. A peak was observed at 3 h in the challenged group (Figure 7.3). For all groups there is a decrease in expression over time except for the control group not exposed to β-1,3/1,6 glucans. 12 h after the *Vibrio sp.* H6 exposure there is a tendency for pathogen exposure to decrease expression levels compared to the non-challenged groups.

7.4. DISCUSSION

Due to the huge range and variability in pathogens, invertebrates need an efficient and quickly adaptable immune defence system. Studies have revealed that highly variable immune

responses can be found in invertebrates as well as evidence that the immune reactions highly depend on the pathogen involved (Cerenius & Söderhall, 2013). Immunostimulants are already globally used in aquaculture as an alternative strategy in the combat against disease. Several studies have already demonstrated that these compounds can indeed heighten the immune system of animals during a certain period of time, most often hours to days, using certain immune indices (Smith *et al.,* 2003). Only few data have been presented to clarify their effects at the molecular level. Gene transcription and protein expression are accepted methods to evaluate the effectiveness of these immunostimulatory compounds on the defence system of the host and its protective effect against pathogens. Nevertheless, increases in gene transcription or protein amounts should only be used as an indication not an absolute, given that some proteins and genes are not regulated after infection depending on the pathogen used (Hauton *et al*., 2014). In this study we assessed how a treatment with β-glucans and/or a challenge with the virulent *Vibrio* sp. H6 affected the mRNA expression level of several innate immune genes in *Artemia*. In addition, we evaluated how a pre-exposure of *Artemia* with β-glucans influenced the *Vibrio*-induced mRNA expression pattern. Based on the literature we selected eight genes involved in innate immunity in invertebrates, including the Down Syndrome Cell Adhesion molecule, masquarade-like protein, lipopolysaccharide and β-1,3-glucan binding protein, peroxinectin, transglutaminases, prophenoloxidase, extracellular superoxide dismutase and heat shock protein 70. For this chapter the pathogens were added immediately after the washing step based on results obtained in Chapter 6. Survival was high for both the treated groups as the control at this time point, therefore it could provide us a good first insight into the varation in gene expression. Further experiments at the other time points are recommend for future research.

When looking at the overview with the significant changes in the mRNA expression levels (Table 7.3) we can notice several things: some genes are very much affected by the treatment with β-glucans and a challenge with the virulent *Vibrio* sp. H6; the effect of β-glucans on the *Vibrio* challenge is noticeable in some of the genes, while others do not show any significant change in expression over the time frame in this experimental study. Changes in gene expression between a 0.5 and 2-fold threshold are often not considered as biologically relevant (thresholds are indicated in the graphs).

No significant changes in expression levels were observed for *dscam*, *hsp70* and *tgase*. The lipopolysaccharide and β-1,3-glucan binding protein (LGBP), also known as Gram-negative bacteria-binding protein (GNBP), is a pattern recognition receptor originally purified from the silkworm (*Bombyx mori)*. It recognises and binds both LPS and β-1,3/1,6 glucans present in Gram-negative bacteria and fungi. Previous studies using the scallop *Chlamys farreri* and the shrimp *Fenneropenaeus chinensis* showed up-regulation of the gene post-stimulation with LPS and β-glucans. In *F. chinensis* this increase was observed 6 h post *Vibrio anguillarum* infection (Yang *et al*., 2010; Liu *et al*., 2007). In another study, *lgbp* transcripts were increased 24 h after infection of the shrimp *Penaeus monodon* with *Vibrio harveyi* (Amparyup *et al*., 2012). Results presented here showed significant down-regulation of *lgbp* expression levels 6 h under influence of the exposure to *Vibrio* sp. H6 (Table 7.4). Yet, no significant influence on gene expression was detected due to the β-1,3/1,6 glucan contact.

Table 7.4: Overview of the statistically significant results for all immune-related genes tested in this study in *Artemia franciscana*. The effect of β-glucans and/or a challenge with the virulent *Vibrio* sp. H6 and how a pre-exposure with β-glucans influenced the *Vibrio*-induced mRNA expression pattern are shown. **Legend:** G**reen**, significant up-regulation of the gene compared to the control at this time point; **Blue**, significant down-regulation of the gene compared to the control at this time point; **-**, no significant difference between the treatment and the control at this time point

	Blanco vs. β-glucans				**Blanco vs. H6**			**Blanco vs. β-glucans + H6**		
	0 h	3 h	6 h	12 h	3 h	6 h	12 h	3 h	6 h	12 h
dscam	-	-	-	-	-	-	-	-	-	-
tgase	-	-	-	-	-	-	-	-	-	-
hsp70	-	-	-	-	-	-	-	-	-	-
lgbp	-	-	-	-	-	0.49	-	-	0.47	-
masq	-	3.38	-	-	9.58	339.16	90.74	5.84	223.65	-
esod	0.16	0.51	3.44	0.01	-	0.13	-	-	0.17	0.02
pero	-	-	0.62	1.32	-	0.42	-	-	0.51	1.03
proPO	-	-	1.72	-	-	-	-	-	-	-

The masquarade-like protein can bind to bacteria and functions as a pattern recognition receptor and cell adhesion molecule in crayfish. It has been suggested that this is an immune inducible gene associated with bacterial infection. Indeed, upon infection of *Peneaus monodan* with *V. harveyi* qPCR analysis showed an increased mRNA expression of the masquarade like serine proteinase inhibitor (*PmMasSPH*) 24 h post infection (Amparyup *et al.*, 2007). Our results showed that the β-glucans, a challenge with the virulent *Vibrio* sp. H6, and the combination of both treatments affected the mRNA expression levels of *masq* significantly (Table 7.3). These results should be interpreted with care though, since the primer developed by Dechama *et al*. (unpublished) for the masquarade-like protein might not detect the correct gene in view of the low homology with masquerade-like protein for instance for the one of *Pacifastacus* (see Appendix). Homology search indicates that the peptide has a Serine proteinase stubble activity, yet there is no confirmation that it is masquarade-like protein. Serine proteinase stubble is a hormone dependent protease required for epithelial morphogenesis, e.g. the formation of appendices.

Extracellular superoxide dismutase (eSOD) plays a major role in antioxidant defence mechanisms and is present in all crustaceans (Mohankumar & Ramasamy, 2006; Yao *et al.*, 2004; Brouwer *et al*., 2003; Orbea *et al*., 2000; Fridovich, 1995). Increases in its expression levels have often been considered by researchers as an indicator for a positive stimulation of the immune response of the host animal. In *Litopenaeus vannamei* the *esod* transcription increased rapidly 1 h after a challenge with the White spot syndrome virus (Gómez-Anduro *et al* 2006). However high superoxide dismutase levels are correlated to the detoxification of the host's body from reactive oxygen species produced when animals are stressed. These ROS are detrimental to the animal's homeostasis (Hauton *et al*., 2014). Cheng *et al.* (2006) showed that *esod* transcription decreased after a *Lactococcus garvieae* challenge in *Macrobrachium rosenbergii*. Our results also showed a significant down-regulation of *esod* expression under influence of β-glucans at time zero, 3 h and 12 h. For these last two time points, expression levels were also significantly lowered in the presence of the combination of β-glucans and pathogen. Therefore, these results suggest that β-glucans did effectively protect *Artemia franciscana* upon a challenge with H6 especially 6 h after challenge.

Peroxinectin, a multifunctional protein of the proPO system, is synthesised and stored in an inactive form. Upon stimulation these proteins become activated (Johansson *et al.*, 1988;

Liang *et al*., 1992). The multiple functions of this protein involve cell adhesion, opsonification, degranulation, peroxidase activity and encapsulation enhancement (Johansson *et al.,* 1988; Thörnqvis *et al*., 1994; Johansson *et al.,* 1989; Johansson *et al*., 1995; Kobayashi *et al.,* 1990). Results showed a significant effect on expression in the presence of the β-glucans, the *Vibrio* sp. H6 and the combination of both. H6 induced a significant upregulation at 6 h which maintained at 12 h, though not statistically significant at this time point. A significant up-regulation also occurred at time point 6 h followed by a significant down-regulation at 12 h. The same trend was observed when both the β-glucans and the *Vibrio* sp. H6 were administered together. These lower expression levels indicate that the β-glucans have a protective effect (based on survival data of Chapter 6). They induce a down-regulation of the *pero* mRNA expression levels and reduce the need for its protein. The exposure to the β-glucans protected the host upon a challenge with H6.

The natural biological activity of peroxinectin is also associated with activation of the proPO system (Johansson *et al.,* 1995). The prophenoloxidase (proPO) activating system is important in the crustacean immune system as it is one of the major immune defence mechanisms against several pathogens for invertebrate animals (Cerenius and Söderhäll, 2004). The expression pattern of proPO in this study indicated that the β-1,3/1,6 glucans induced proPO expression (at 6 h), however this effect was not observed in case of a simultaneous exposure to *Vibrio*. This might indicate that the beneficial effect of the β-1,3/1,6 glucans on *Artemia* survival upon H6 challenge is not dependent on an increased *proPO* expression. Furthermore, when comparing the expression values of *proPO* with those of *pero*, we can see the absence of a link between the two. If *pero* would only serve as an activator of the proPO cascade then consecutive expression patterns could be anticipated. This is not the case, indicating that their connection is more complex.

To determine dynamic changes in mRNA expression levels, time-dependent measurements are necessary. Unfortunately, due to practical difficulties not enough biological replicates were sampled to perform adequate statistical tests to validate these dynamics over time. Nevertheless some trends could be observed. In the present study, expression values for the peroxinectin control were quite high at 12 h indicating that the host might have experienced physiological stress at this time point or alternatively the ingestion of LVS3-particles might have stimulated the *pero* expression. So the addition of the β-glucans

normalises the effects. The *masq* expression demonstrated overall a very strong dynamic response over the experimental period, including in the blank. At the moment it is not clear what the underlying cause might be. Probably, this expression profile could be linked to the accumulating ingestion of LVS3-*Aeromonas* particles. To verify this, an experiment would need to be set-up comparing starving *Artemia* with LVS3-fed *Artemia*.

This is the first time all these genes have been investigated in a gnotobiotic *Artemia franciscana* system at once. Overall, we can say that there are no uniform patterns in expression levels; the least to be said is that reactions are multifaceted. This complexity can be due to several things and conclusions should be drawn with care. One proposed hypothesis is that the gene dynamics are very variable and difficult to portray with 3 h time intervals. Hence, the sampling points chosen in this study might not have monitored the gene expression within the proper time frame or interval. Alternatively, translational control and enzyme activity might be more important than transcriptional control. Also, in the present study, expression levels sometimes demonstrated overall a very strong dynamic response over the experimental period, including in the blank. A possible explanation could be provided by the accumulating ingestion of LVS3-*Aeromonas* particles. *Aeromonas hydrophila* is itself a Gram-negative bacterium, which therefore could stimulate the immune system of the host by itself. Observed results could therefore be skewed due to interference of these LVS3-particles. Finally, another hypothesis that has been put forward is that expression profiles of genes of the host animals are very pathogen specific (Hauton *et al*., 2014). Therefore results obtained in this PhD thesis might very well be *Artemia*-*Vibrio* sp. H6 specific and hence difficult to compare with expression profiles in other host-*Vibrio* interaction models.

The focus of this study has been on mRNA expression levels of eight selected genes correlated with the immune responses in invertebrates. Nevertheless, it shouldn't be forgotten that other innate immune mechanisms might be more important in the defence against bacteria. mRNA expression levels do not offer us all the answers and they should be viewed in a more holistic approach towards immune responses in invertebrates.

It is also worth to be noted that this study was a preliminary study for five of the researched genes (*dscam, masq, pero, lgbp* and *esod*). These genes were newly identified for *Artemia franciscana* only on the basis of homology between crayfish and the *Artemia* genome

database (see Appendix). The *Artemia* genome currently available still contains too many genes, most probably because a bulk sequence on siblings was performed. Also the biochemical activity of the genes identified by homology should be confirmed by proper biochemical assays, which requires cloning and expression of the genes in an appropriate expression system. Hence, the mRNA expression levels observed in this study should be interpreted with care since the functionality of these newly identified genes has not been confirmed by further research.

7.5. CONCLUSION

In this study, the effect of β-1,3/1,6-glucans and a challenge with the *Vibrio* strain H6 on the gene expression of eight innate immune genes was characterised in a gnotobiotic *Artemia* system. Altogether, the obtained results suggest that in *Artemia franciscana* β-1,3/1,6 glucan particles can stimulate the innate immune system by modulating the expression of immune-related genes either as such or in combination with a *Vibrio* challenge. These β-glucans also provide protection to the animals upon exposure to a pathogen. Further research is however recommended to clarify the mechanistic link between these two phenomena despite the fact that well-defined gnotobiotic conditions were used. Exploration of other immune mechanisms is recommended as well, in order to obtain a more overall picture of immune responses in crustaceans.

CHAPTER 8

GENERAL DISCUSSION & CONCLUSION

CHAPTER 8: General discussion & conclusion

With an annual production of 6.4 million tonnes and a value of US$ 30.9 billion (FAO, 2014), cultivation of crustaceans is one of the most profitable sectors within aquaculture (Smith *et al*., 2003). Progress and sustainable production remains mostly hampered by insufficient control of infectious diseases and pathogens, mainly at the larval life stages. Due to the occurrence and spread of antibiotic resistant pathogens other strategies to control diseases are needed to obtain a more sustainable production (Defoirdt *et al*., 2007; ECDC, 2014). One promising group of disease control agents are immunostimulants and since they naturally occur in the environment, their use raises less concern (Miest & Hoole, 2014). A proper validation of their efficacy, a more thorough knowledge on their short- and potential long-term effects on the host organisms, an understanding of the underlying mechanisms of host-microbe interactions and pathogen infection are necessary. This can be obtained through standardised trials and a good model system. This highlights the importance of the general aim of this PhD study, i.e. to validate the immunological response of *Artemia franciscana* after exposure to β-1,3/1,6-glucans together with a challenge by a pathogenic bacterium.

Gnotobiotic model systems offer a unique way to study host-microbe interactions without interference of unknown microbial communities that are naturally present in the rearing environment (Figure 8.1). Larvae of the brine shrimp *Artemia*, an important live-food in fish and shellfish larviculture, are interesting model organisms. Being small animals with a short life cycle, they allow for an easy integration of experimental approaches and molecular analyses (Sung *et al*., 2008). A well-characterised gnotobiotic *Artemia* rearing system (GART) has been developed, and has previously been used to study nutritional or immunostimulatory properties of yeast (Marques *et al*., 2006), algae (Marques *et al*., 2004) and bacteria (Marques *et al*., 2005). Until now, however, the GART system could not be maintained long enough to study nutritional or immunostimulatory effects over a longer period of time or during the whole life cycle of the organism. In order to validate the immunological response of *Artemia franciscana* after being exposed to β-1,3/1,6-glucans, two lines of research were developed in this PhD study. The first goal was optimizing the gnotobiotic *Artemia* rearing system for ultimately monitoring long-term effects of immunostimulants and infection. Secondly, experiments were designed to determine the

mechanism by which β-glucans affect *Artemia franciscana*, with respect to both the gastrointestinal tract morphology and the activity of the immune system.

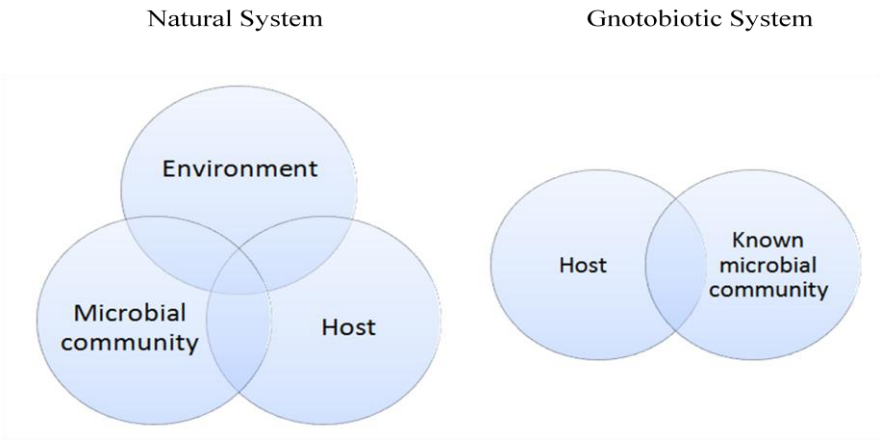

Figure 8.1: A schematic representation of host-pathogen interactions in a complex natural system (left) vs. a gnotobiotic system (right)

8.1. *ARTEMIA FRANCISCANA* & *VIBRIO* sp. H6

Vibriosis is a common disease causing major economic losses in crustacean rearing (FAO, 2012). Opportunistic *Vibrio* strains are the evildoers, mainly *V. harveyi*, *V. campbellii* and *V. parahaemolyticus*. Opportunists can survive and multiply outside their host. They can more easily infect their hosts when they are immune suppressed or otherwise physiologically stressed due to poor environmental quality by intensive culturing. Symptoms include lethargy, tissue and appendage necrosis, slowed growth, body malformation and bioluminescence of the infected host (Aguirre-Guzman *et al.*, 2004). The known human pathogens belonging to the *Vibrio* genus (i.e. *Vibrio cholera*, *V. parahaemolyticus* and *V. vulnificus*) are susceptible to resistance against antibiotics due to horizontal transfer from the resistant marine strains originating from the misuse of antibiotics occurring.

The strain that until now has routinely been used in brine shrimp challenge tests at the lab (*Vibrio campbellii* LMG 21363) is not virulent enough towards older animals in order to enable long-term studies. Therefore, a more virulent strain was needed, which was found in the newly identified *Vibrio* strain H6 (**Chapter 3**). Results presented in this PhD thesis

showed that H6 induces a significant higher mortality both in gnotobiotic and conventional *Artemia* than *V. campbellii* LMG21363. When *Vibrio campbellii* LMG 21363 is used to challenge instar II nauplii, survival is typically around 20% after 2 days of challenge. Yet, when *Vibrio* sp. H6 is used, survival is as low as 5% after 48 h. To our knowledge, H6 is the most virulent strain described for the gnotobiotic *Artemia* model system thus far.

The preliminary genetic phenotyping performed on the *Vibrio* sp. strain H6 (Figure 3.3) found species of the *Vibrio harveyi* group as the closest relatives and the highest pairwise sequence similarity in this group corresponded to *Vibrio natriegens* (99.1%; Yoshizawa *et al*., 2009). *Vibrio natriegens* is a fast-growing bacterium with a generation time of less than 10 min (Eagon, 1962), nonetheless this was not the case for *Vibrio* sp. H6. It is a Gram-negative species isolated from salt mud marshes. It has been reported to be nonpathogenic (Aiyar *et al*., 2002) and occurs as intestinal microbiota of sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) when the larvae were fed with *Artemia* (Grisez *et al*., 1997). Despite the resemblance between *Vibrio* sp. H6 and *V. natriegens*, there are some major differences between their growth rate and their pathogenicity towards *Artemia*, indicating that it might be interesting to verify if H6 is a new *Vibrio* species, using a polyphasic approach (Thompson *et al*., 2009).

8.2. MICROALGAE & THE GNOTOBIOTIC *ARTEMIA* SYSTEM

The current gnotobiotic system using *Artemia franciscana* can be maintained for 2 days using dead *Aeromonas* sp. LVS3 cells as feed and for 6 days using dead *Aeromonas* sp. LVS3 in combination with special yeast cells such as mnn9 (Marques *et al.*, 2004). To investigate long-term effects of β-glucans on *Artemia franciscana* however, the gnotobiotic rearing period has to be prolonged beyond the current time frame. During this PhD study, we evaluated axenic microalgae as a feed to prolong the rearing period. This was done by monitoring survival and by a comparative study of the histology and cellular morphology of the alimentary tract of gnotobiotic *Artemia* nauplii.

This PhD work showed that axenic microalgae did not allow rearing of gnotobiotic *Artemia franciscana* until the adult stage since the survival after 5 days was too low. A

comparison of the digestive tract of gnotobiotic and conventionally grown animals revealed severe deficiencies in the morphology of the gnotobiotic animals already in the early stages of their life. Our results indicated that until 2 days of rearing, the internal development is comparable between the gnotobiotic and conventional animals (**Chapter 4**). On the contrary, the deteriorated state of the gastrointestinal tract of the axenic animals at day 4 is not deniable. Fewer cell organelles, more vacuolisation and fewer microvilli in the brush border are clear indications that the cell morphology was disturbed in gnotobiotic animals. Moreover, several lysosomes were observed in midgut epithelial cells of gnotobiotic *Artemia* (Figure 4.6 E). Lysosomes contain hydrolytic enzymes and are produced by the Golgi-apparatus. They are considered to be the catabolic tools of the cells. When animals are under physiological stress, an accumulation of lysosomes in the cells can occur, also known as autophagy (Rekecki, 2012). This observation together with the deteriorated status of the brush border at day 4 (**Chapter 4**) leads us to hypothesise that the gnotobiotic animals did not succeed in absorbing sufficient nutrients to maintain their homeostasis.

Figure 8.2: 4-day old conventionally reared nauplius of *Artemia franciscana* from Great Salt Lake

8.3. β-GLUCANS & THEIR PROTECTIVE EFFECT AGAINST *VIBRIO* SP. H6

The second part of this PhD work dealt with the effect of β-1,3/1,6-glucans both on survival (**Chapter 6**) and immune gene expression (**Chapter 7**) of *Artemia franciscana*. Commercial β-1,3/1,6-glucan particles derived from a specially-selected *Saccharomyces cerevisiae* strain (MacroGard) were used. The survival of the *Artemia* nauplii was determined 2 days subsequent to a 6 h oral stimulation period with the β-glucans followed by inoculation of *Vibrio* sp. H6 into the rearing water. A significant increase in survival was demonstrated following exposure to β-1,3/1,6-glucans (Chapter 6).

The efficacy of β-1,3/1,6-glucans (MacroGard) on the survival of various cultured animals has been reported already in earlier studies (Miest & Hoole, 2014). Immersion of black tiger shrimp (*Penaeus monodon*) post-larvae in a suspension of MacroGard increased growth performance, immune response and disease resistance (Supamattaya *et al*., 2000). Since the effect of immunostimulatory compounds is very much host-pathogen specific (Ai *et al.*, 2007), the effect of β-glucans on the survival of *Artemia* was re-evaluated in combination with *Vibrio* sp. H6. Results obtained in **Chapter 5** showed that a challenge with *Vibrio* sp. H6 was no longer effective after day 5; a phenomenon previously observed with *V. campbellii* as well. Therefore, evaluating the effects of the immunostimulating effects of β-glucans on *Artemia franciscana* was focused on the earlier life stages in the following chapters of this PhD dissertation.

In order to administer the β-glucans to the *Artemia*, a bath immersion strategy was used here as described by Soltanian *et al.* (2007). A dose-response relationship is often non-linear, when low doses do not have any effect, higher doses can be negative for the host's physiology (Sung *et al*., 1994; Hauton *et al*., 2014). The particle uptake rate for 2 day old *Artemia* nauplii has been calculated by Coutteau *et al.* (1992), who reported that an increase in concentration of yeast is accompanied by a decrease in the uptake rate (Coutteau *et al.*, 1992). Further research is needed to determine the uptake of immunstimulatory compounds by the *Artemia* in the gnotobiotic rearing system.

The glucan exposure time of 6 h (**Chapter 6 & 7**) was used based on previous studies performed at the Laboratory of Aquaculture & *Artemia* Reference Center (Niu *et al*., 2014) and preliminary experiments performed in the framework of this PhD study. Similar to what had been found for other vibrios (Niu *et al*., 2014), the addition of β-glucans at the same time of challenge with *Vibrio* sp. H6 did not have any effect on survival. While an exposure time of 12 h resulted in a survival of around 50% after challenge with *Vibrio* sp. H6. Survival with a 12 h exposure time was lower than with the survival after a 6 h exposure period.

To exclude interference of the β-glucan feeding effect on the survival, relative percentage of survival (RPS) values were calculated (Figure 8.2; representation of data from Chapter 3). The RPS values illustrate the duration of the protective effect of β-1,3/1,6 glucans on *Artemia* survival. A protective effect of the β-glucans is visible over the whole duration of the experiment, although only significant until 6 h post glucan removal (**Chapter 3**). The used concentration is not detrimental to the host, but rather provides a nutritional benefit for the animals together with protection against the pathogen H6.

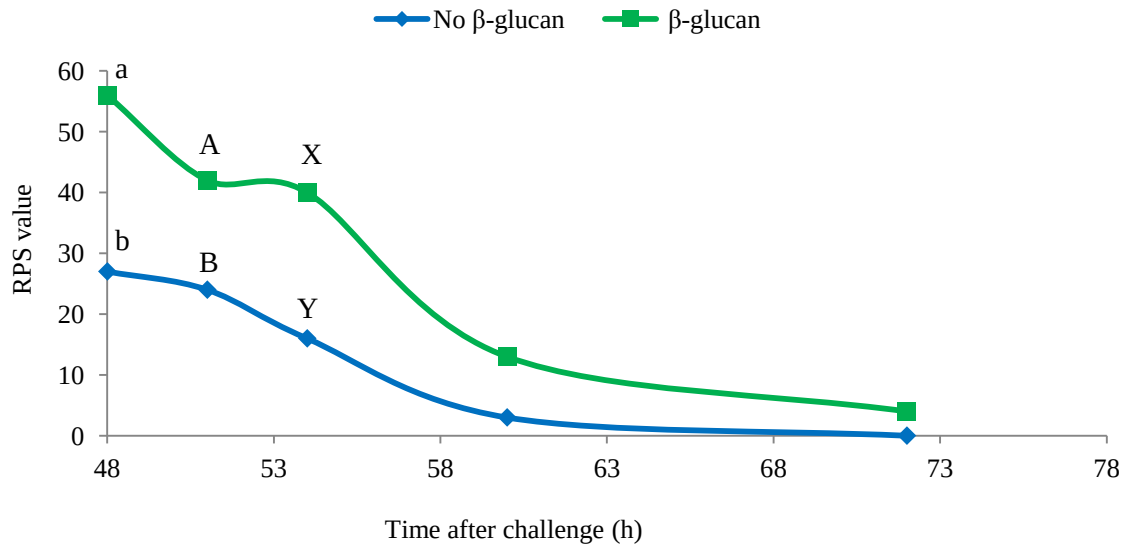

Figure 8.2: Effect of pre-treatment with MacroGard compared to the control group. Relative percentage of survival (RPS) is shown over time of gnotobiotic brine shrimp larvae. Data with different superscripts are significantly different in a one-way ANOVA analysis ($P < 0.05$).

In this study, priming is defined as a phenomenon whereby a strong synergistic immunological response is generated by a combined or subsequent exposure to an

immunostimulant and a pathogen. We therefore cannot conclude that the pre-treatment of the *Artemia* during 6 h with β-glucans, effectively primed their immune system. We can conclude though that MacroGard, at a concentration of 20 mg l^{-1}, offers protection to *Artemia* when exposed to *Vibrio* sp. H6.

8.4. β-GLUCANS & THEIR IMPACT ON IMMUNE GENES EXPRESSION IN *ARTEMIA FRANCISCANA*

More and more information is gathered these days about how pathogen infection and immunostimulants affect gene expression in crustaceans (Hu *et al*., 2014), yet not much is known concerning *Artemia franciscana.* Studies focusing on immune responses in invertebrates proved to produce inconsistent results upon stimulation (Hauton *et al.*, 2014). In experimental research, mRNA expression levels have often been used as measures for immune responses in different animals in the presence of stimulating compounds and pathogens. The assumption is that mRNA transcript quantities determine protein abundances. Yet, mRNA expression levels do not always correlate to the actual protein concentrations present in the animal (Greenbaum *et al*., 2003). There are arguments for a strong regulatory role for processes downstream of transcription (Vogel & Marcotte, 2012). A clear correlation between mRNA expression levels and protein activity has not been determined yet. According to Greenbaum *et al*. (2003) the final concentration of proteins is really what we should be studying instead of mRNA expression levels since that will influence the host responses in the end. Absolute quantities of proteins can now be determined using proteomics, two-dimensional electrophoresis and mass-spectrometric methods (Vogel & Marcotte, 2012). A reason for the poor correlation between mRNA and protein levels has been put forward in literature. Greenbaum and colleagues (2003) stated that for genes with a strong regulatory control at mRNA expression level, there is minimal control at the final protein quantity. On the contrary, when there is minimal variation in mRNA expression the final protein level would be controlled at the post-translational level, with little or no correlation between mRNA expression and protein levels.

In this dissertation, for the first time, the expression of eight genes, five of which were newly identified genes (*dscam, masq, lgbp, pero* and *eSOD*) for *Artemia franciscana,* was

verified upon inoculation with the novel *Vibrio* strain H6 (**Chapter 7**). The small-scale culturing system of *A. franciscana* under gnotobiotic conditions allows for a rapid screening of gene expression in the presence of a pathogen. Results indicate that β-1,3/1,6-glucans are a promising way as an alternative strategy to combat disease in aquaculture. The studies on the effects of β-glucans on the gene expression in *Artemia franciscana* are still preliminary though and remain to be further explored.

For most animals with available genomes, several copies of the *dscam* gene were found (Brites *et al*., 2008; Chou *et al*., 2011). This cell membrane molecule consists of a cytoplasmic tail and an extracellular region, which is very variable. It could play a role in specific pathogen recognition and the observed phenomenon of priming (Chou *et al*., 2011; Watthanasurorot *et al*., 2011). Nevertheless, our results did not suggest any significant changes in expression levels upon a challenge or β-glucan exposure. Besides Dscam we also analysed the mRNA expression of a masquarade-like protein. Usually, this protein functions as a prophenoloxidase activating factor, however, in crayfish (*Pacifastacus leniusculus*) it functions as a PRR and cell adhesion protein (Amparyup *et al.*, 2007). Therefore, further molecular characterisation of the masquarade-like protein identified in *Artemia franciscana* should be performed in order to elucidate the function of this molecule. Notwithstanding, our results indicate a role of the masq-like protein in crustacean immunity as the presence of the H6 strain induced an immense increase in mRNA expression levels at 6 h post challenge as compared to the control group. This corresponds to observations made by Kwon *et al*. (2000), who researched the expression of *H. diomphalia* masquarade-like Serine Protease Homologue (45 kDa SPH) mRNA after *E. coli* injection. They found up-regulated mRNA expression levels 8 h after injection which persisted for 48 h.

In crayfish (*P. leniusculus*), peroxinectin has a cell adhesion function together with peroxidase activities induced by β-glucans (Johansson & Söderhäll, 1988). Liu *et al*. (2005) showed an up-regulation in peroxinectin transcription 6, 12 and 24 h post *Vibrio alginolyticus* injection. In the Pacific white shrimp *Litopenaeus vannamei*, a sublethal dose of *Vibrio campbellii* significantly increased peroxinectin levels 48 h following injection (Burge *et al.*, 2009). However, in both studies the pathogens were injected into the animals, whilst in our study an emersion technique was used. At 12 h, β-glucans significantly down-regulated expression levels even in the presence of a pathogen, while the values for the challenged groups were up-regulated at the same time point. These lower expression levels indicate that

the host is protected against stress by the exposure to the β-glucans. It must be noted that there is still uncertainty concerning the obtained *pero* sequence as homologies were detected with peroxidasin, which is related to peroxinectin. Further validation of this sequence is certainly warranted.

Superoxide dismutases are biomarkers for immune stimulation and disease in crustaceans (Tian *et al*., 2011). They are necessary to remove high levels of reactive oxygen species from the host's body. In *L. vannamei* the eSOD transcription level increased rapidly 1h after white spot syndrome virus (WSSV) challenge (Gómez-Anduro *et al* 2006). In the study by Campa-Cordova *et al*. (2002) a twofold increase was observed in hemocytes compared to the control after stimulation of the juveniles of American white shrimp (*L. vannamei*) with β-glucans and sulphated polysaccharide solutions. Results obtained in this study correspond to these observations; a peak in transcription occurred 3 h after the *Vibrio* challenge, while β-glucans induce lower expression levels. From 6 h onwards there is an overall decline in expression levels. Results suggest that β-glucans did effectively protect *Artemia franciscana* upon a challenge with H6.

Sivakama & Vaseeharan (2012) reviewed the characterisation and classification of lipopolysaccharide and β-1,3-glucan binding protein (LGBP) in decapod crustaceans. LGBP is activated upon bacterial and fungal infection and activate in their turn the prophenoloxidase cascade (Lee *et al*., 2000; Roux *et al*., 2002). Sivakama & Vaseeharan (2012) showed that the expression of the *lgbp* gene was up-regulated in hemocytes 6 h following a challenge with peptidoglycans and *Vibrio parahaemolyticus*. In the present study, opposite mRNA expression levels were detected upon infection. β-glucans significantly down-regulated *lgbp* expression in the presence of *Vibrio* sp. H6.

8.5. CONCLUSION

The results presented in this PhD study demonstrate fundamental insights into the proposed research topics. First of all, histological research showed that in order to research the effects of immunostimulation throughout the entire life span of gnotobiotic *Artemia franciscana* another appropriate feed has to be found to be able to prolong the gnotobiotic

culture. Animals fed with the axenic microalgae *Tetraselmis suecica* had a poorly developed gastrointestinal tract which led to exhaustion and death of the animals after 5 days. Stimulation with β-glucans did significantly increase the survival of *Artemia franciscana* nauplii upon a challenge with the pathogenic bacterium *Vibrio* sp. H6. The protective effect of the β-glucans (MacroGard) at a concentration of 20 mg l^{-1} lasted for at least 6 h. No effective proof of priming with respect to gene expression was found under the described culture conditions when the β-glucans were removed from the experimental system after a 6 h exposure period.

Further research on immune-related gene expression, as well a protein production and enzyme activity is recommended. Results obtained in this PhD study might be very much host-pathogen specific, nevertheless they provide a new outlook into the immunological research of responses of *Artemia* subjected to immunostimulation in combination with exposure to a pathogen.

8.6. FUTURE PERSPECTIVES

Artemia franciscana & Vibrio sp. H6

Results obtained in this PhD are just the foundation for describing this new emerging and highly virulent pathogen. Further identification and characterisation of these and other virulence factors is warranted. Gathering information on the biofilm formation and quorum sensing of H6 might provide further insight in the pathogenesis and offer an explanation for the observed virulence since no explanation could be found up to now. *Vibrio* taxonomy has been based on a polyphasic approach yet in this study only the 16sRNA gene sequences were determined. To obtain a better molecular phylogeny, whole-genome sequence based analyses are recommended together with Multilocus Sequence Analysis (MLSA) or Average Amino Acid Identity (AAI) for example. Determination of the GC ratios is another option, as are further phenotypic analyses, as for example fatty acid analysis. The more information about this possibly harmful pathogen for the aquaculture sector can be acquired, the more suitable ways to combat it can be devised.

Microalgae & the gnotobiotic Artemia system

Based on the results obtained in this PhD study, the question can be raised whether it is just a matter of finding the appropriate feed source or whether it is at all possible to grow *Artemia franciscana* until the adult stage under gnotobiotic conditions free of bacterial cells, either dead or alive. In future experiments other feeds will be tested in the gnotobiotic *Artemia franciscana* system, for example other strains of axenic microalgae in combination with dead bacteria or previously used yeast strains (Marques *et al*., 2004). The combination of axenic *Artemia* fed with gnotobiotic algae should be further investigated to determine the effect of the presence of bacteria in the algae culture on the growth and internal development of *Artemia*. Preliminary tests have already been performed during this PhD and the first results proved promising but further experiments are necessary to be able to draw sound conclusions on the topic. The presence of symbiotic bacteria in the gastrointestinal tract might be required for the animals to reach adulthood. Similar observations have been made in zebrafishes (Bates *et al.,* 2006*;* Rawls *et al.,* 2004). Germ-free animals showed first a stop in the differentiation of the gut epithelium and a lack of microvilli brush border. Yet when they were transferred to conventional conditions all of the germ-free phenotypes were reversed under influence of the presence of a single bacterium or a complex of microbiota (Bates *et al.*, 2006).

***β-glucans & their protective effect against Vibrio* sp. H6**

MacroGard has already been used in a large scale farming environment. A concentration of 20 mg l^{-1} proved not to be detrimental and is feasible on a large scale culture system. However, results showed that an exposure time of 6 h protected *Artemia* during 6 h against pathogens. Accordingly, we hypothesised that if this concentration would be administered in a farm environment using the same exposure time, they would have to be added regularly during to the culture set-up, i.e. every 6 h. Extrapolation from well-constrained host/pathogen model systems in laboratory studies to a large-scale farm set-up in crustacean aquaculture proves challenging. The obtained results might be very host-microbe specific to the *Artemia franciscana* from Great Salt Lake and *Vibrio* sp. H6 model. Although the survival results have been confirmed using the San Francisco strain of *Artemia* with *Vibrio* sp. H6, further validation of the obtained data should be done using other host-microbe models.

β-glucans & immune gene expression in Artemia franciscana

The primers for the five newly identified genes were designed on the sequence homology between crayfish and the *Artemia* genome database. Yet further validation of the biochemical activity of these genes is recommended as well (see Appendix). The mRNA expression levels should be interpreted with care since the functionality of these newly identified genes has not yet been confirmed. Their biochemical characteristics, expression patterns and their role in the innate immune system of *Artemia franciscana* require further validation and research. Complementation, microarrays, molecular cloning and verification on protein level can be carried out too in future research. Molecular cloning can be used for researching the expression of a specific gene. By inactivation or mutation the function of a particular gene and its correlation with immunological responses can be explored. With complementation researchers can determine whether or not a certain phenotype can be assigned to a particular gene, even without prior knowledge of the gene's functioning. Confirmation of the obtained expression results should be confirmed and verified on the protein level as well.

One observation that is clearly visible from the gene expression results obtained in this PhD thesis is that the gene expression is highly variable and changing extremely quickly. Studies done with only one sampling point at a certain time during the experiments only provide a snapshot of the true variability and unpredictability after stimulation of these immune genes.

APPENDIX

Homology data for the five newly identified genes in *Artemia franciscana*

<table>
<tr><th colspan="2">esod</th></tr>
<tr><td>DNA amplified by primer</td><td>2 ggtggtccagatgatacgaggcgccatgtcggagatcttggaaac
 G G P D D T R R H V G D L G N
47 attcaatttgtagaagaaccaactgaagaagctgcagtggcaaga
  I Q F V E E P T E E A A V A R
92 attatcatcgaagacacagaaatatccctatgcggagaaaga
  I I I E D T E I S L C G E R</td></tr>
<tr><td>ORF in the Artemia database</td><td>MVFLPEEHGGPDDTRRHVGDLGNIQFVEEPTEEAAVARINIEDSEISLCGERNVIGRAIVVHAGPDDL
GRGRNEESQKTGNAGPRAGCGIVEETEGLLDSSEIF</td></tr>
<tr><td>Sequence of Pacifastacus leniusculus</td><td>MVNMTLPDMLVKMMIVGIMSFMALASPPAPAAVVDLVPGSDQISGRLEIYRSYNGLTIVGTVSGLTPGKH
GFHVHQKGDLGDGCKAAGGHFNPFNKNHGAPEDLERHAGDFGNVVADYQGVATIYIDDSQVSLDPSSEA
YIGGLAIVVHAGVDDLGRGGNPESAKTGNAGARSGCGIIRVVAPTYQPPQSGYRPRRPQHPNRQPGFPQQF
QYQRTYN</td></tr>
<tr><td>Homology with Pacifastacus leniusculus</td><td>
Artemia 1 ---------- 0
Pacifastacus 1 MVNMTLPDMLVKMMIVGIMSFMALASPPAPAAVVDLVPGSDQISGRLEIY 50
Artemia 1 ----------MVFLP--EEHGG 10
Pacifastacus 51 RSYNGLTIVGTVSGLTPGKHGFHVHQKGDLGDGCKAAGGHFNPFNKNHGA 100
Artemia 11 PDDTRRHVGDLGNIQFVEEPTEEAAVARINIEDSEISL--CGERNVIGRA 58
Pacifastacus 101 PEDLERHAGDFGNV-----VADYQGVATIYIDDSQVSLDPSSEAYIGGLA 145
Artemia 59 IVVHAGPDDLGRGRNEESQKTGNAGPRAGCGIVEETEGLLDSSEIF---- 104
Pacifastacus 146 IVVHAGVDDLGRGGNPESAKTGNAGARSGCGIIRVVAPTYQPPQSGYRPR 195
Artemia 105 ---------- 104
Pacifastacus 196 RPQHPNRQPGFPQQFQYQRTYN 217
</td></tr>
</table>

<table>
<tr><th colspan="2">masq</th></tr>
<tr><td>DNA amplified by primer</td><td>17 ctgtttcttttagatttcgttcctgaccccactcaagcacaattt
  L F L L D F V P D P T Q A Q F
62 ggcgagttcccttggatggttgtgattcttggccctaacaataac
  G E F P W M V V I L G P N N N
107 tacattggaggcggtgctttgattgacggcaga 139
  Y I G G G A L I D G R</td></tr>
<tr><td>ORF in the Artemia database</td><td>MQIFNDIFSSYSSLLFLLDFVPDPTQAQFGEFPWMVVILGPNNNYIGGGALIDGRHILAQHLYRVHFKSNGG
QGIKVRLGEWDAKQNTEPLKYVEVPVTDIRIHPFFNPANLKNDIALLKLPEAVNFAAAPHVAPVCIPNFAQR
NEGKRQEELIKKLDSSVESIPINYFLEGEGFINKIANSRCWVTGWGKDAFGAGGQFQYILRKVDVPVLHASD
CENRLRSTRLGPIFQLDRTSFVCAGGEPGRDACQVIYSGTNVKSLTLDEASGFLPKSKIGDGGSPLVCETEP
NRFEVVGLVAWGIGCAEPGIPGVYVNVASYVDWIIKEISTP*</td></tr>
<tr><td>Sequence of Pacifastacus leniusculus</td><td>MRSGGLVSILLVVTAGAARAWPQYPGVGSGAGGADELQQTVQGGAKGIAGQQEQGAEGVSLSGDYSFQQA
ADCNKNYGCVPWQLCVDGEINTSGVGQLDLRTPEPVPQKNTATLCAGIGKVCCLLSDHSTTVVEGGGQLP
DSSGNTGSILEVGGGGGALGGGGSAAVVEGTGGSGYQGGGGSTTVVEGAGGALHSSSGTDSIIEAVSGGG
GGSTSVVEGAVGAPHSSGDTGTIVEVGGGGHQAGVGSATAGGASHGSGDTGTIVEVGGGGGHQAGGGSAT
AGGASHGSGDTGTIVEVGGGGGHHAGGGSATAGGASHGSGDTGTIVEVGGGGGHQAGGGSATVGGASHGS
GGTGTIVDVSGGDQHQQSGGKTVVAGQCAETHDCVPTYLCHDGKINTPGEGLLDLRFSSKCVNPNYPTVA
AVCCAYPSCKAGNLCVAHGACEGTIVKDSAGKYEDCFVGPNLDPGICCTPPAPKPLQTCPGPKTCITQPQ
CTARTTFATDGVGNIDIRIHTACFVSKGTVIGVCCDPPAPLEKCSLDGKLKCVASGSCGEQVILDALGEH
QTCYVSGGAGEVGHCCTAPEPLKTCPGGETCLVSDLCHSDGVKASPSNSACYVNPNIVGACCYPAPKPAV
PVHDTCPDKSVCLPEILCQGELLDNTGAFLPYSSSGQWAQCLLSGTGLVSPGVCCENPQLPIPDSTYEAA
DKCGVRNELLDTRIKNNDLLYYQTHFAEFPWQGIIFFTNYTFKCGASLIGDRWLLTAAHCVKGFTPQDLR
VRWVSGRSTSIKSLCSTMMQLWNLLQYIHYLIPKNVHNDIAVIELTEPIVFKYHINTICLPNHGQIIPKG
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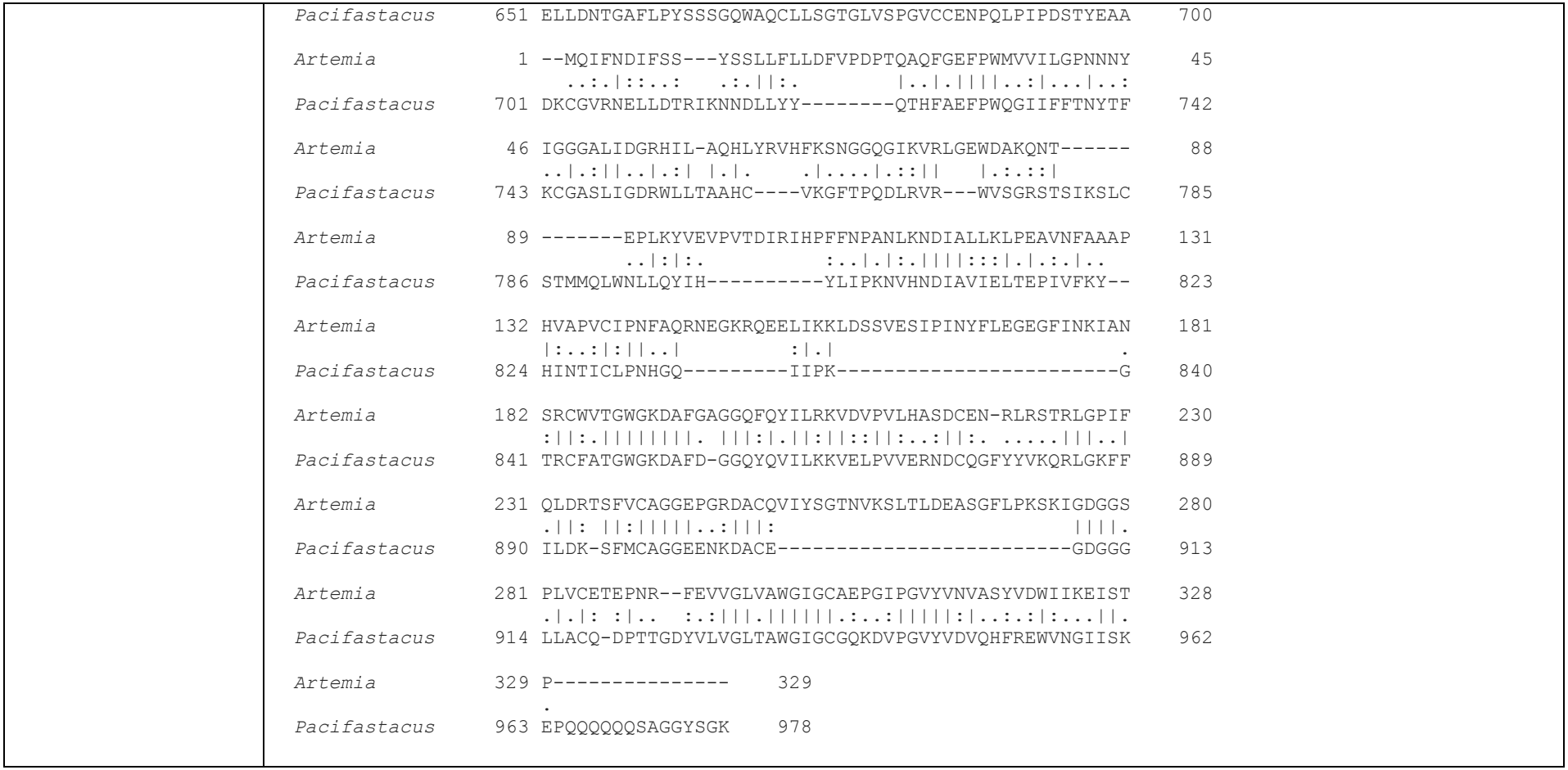
GGGLLACQDPTTGDYVLVGLTAWGIGCGQKDVPGVYVDVQHFREWVNGIISKEPQQQQQQSAGGYSGK</td></tr>
<tr><td>Homology with Pacifastacus leniusculus</td><td>Artemia 1 ---------- 0
Pacifastacus 1 MRSGGLVSILLVVTAGAARAWPQYPGVGSGAGGADELQQTVQGGAKGIAG 50
Artemia 1 ---------- 0
Pacifastacus 51 QQEQGAEGVSLSGDYSFQQAADCNKNYGCVPWQLCVDGEINTSGVGQLDL 100
Artemia 1 ---------- 0</td></tr>
</table>

	Pacifastacus	101	RTPEPVPQKNTATLCAGIGKVCCLLSDHSTTVVEGGGQLPDSSGNTGSIL	150
	Artemia	1	----------	0
	Pacifastacus	151	EVGGGGGALGGGGSAAVVEGTGGSGYQGGGGSTTVVEGAGGALHSSSGTD	200
	Artemia	1	----------	0
	Pacifastacus	201	SIIEAVSGGGGGSTSVVEGAVGAPHSSGDTGTIVEVGGGGHQAGVGSATA	250
	Artemia	1	----------	0
	Pacifastacus	251	GGASHGSGDTGTIVEVGGGGGHQAGGGSATAGGASHGSGDTGTIVEVGGG	300
	Artemia	1	----------	0
	Pacifastacus	301	GGHHAGGGSATAGGASHGSGDTGTIVEVGGGGGHQAGGGSATVGGASHGS	350
	Artemia	1	----------	0
	Pacifastacus	351	GGTGTIVDVSGGDQHQQSGGKTVVAGQCAETHDCVPTYLCHDGKINTPGE	400
	Artemia	1	----------	0
	Pacifastacus	401	GLLDLRFSSKCVNPNYPTVAAVCCAYPSCKAGNLCVAHGACEGTIVKDSA	450
	Artemia	1	----------	0
	Pacifastacus	451	GKYEDCFVGPNLDPGICCTPPAPKPLQTCPGPKTCITQPQCTARTTFATD	500
	Artemia	1	----------	0
	Pacifastacus	501	GVGNIDIRIHTACFVSKGTVIGVCCDPPAPLEKCSLDGKLKCVASGSCGE	550
	Artemia	1	----------	0
	Pacifastacus	551	QVILDALGEHQTCYVSGGAGEVGHCCTAPEPLKTCPGGETCLVSDLCHSD	600
	Artemia	1	----------	0
	Pacifastacus	601	GVKASPSNSACYVNPNIVGACCYPAPKPAVPVHDTCPDKSVCLPEILCQG	650
	Artemia	1	----------	0


Pacifastacus 651 ELLDNTGAFLPYSSSGQWAQCLLSGTGLVSPGVCCENPQLPIPDSTYEAA 700
Artemia 1 --MQIFNDIFSS---YSSLLFLLDFVPDPTQAQFGEFPWMVVILGPNNNY 45
Pacifastacus 701 DKCGVRNELLDTRIKNNDLLYY--------QTHFAEFPWQGIIFFTNYTF 742
Artemia 46 IGGGALIDGRHIL-AQHLYRVHFKSNGGQGIKVRLGEWDAKQNT------ 88
Pacifastacus 743 KCGASLIGDRWLLTAAHC----VKGFTPQDLRVR---WVSGRSTSIKSLC 785
Artemia 89 -------EPLKYVEVPVTDIRIHPFFNPANLKNDIALLKLPEAVNFAAAP 131
Pacifastacus 786 STMMQLWNLLQYIH----------YLIPKNVHNDIAVIELTEPIVFKY-- 823
Artemia 132 HVAPVCIPNFAQRNEGKRQEELIKKLDSSVESIPINYFLEGEGFINKIAN 181
Pacifastacus 824 HINTICLPNHGQ---------IIPK----------G 840
Artemia 182 SRCWVTGWGKDAFGAGGQFQYILRKVDVPVLHASDCEN-RLRSTRLGPIF 230
Pacifastacus 841 TRCFATGWGKDAFD-GGQYQVILKKVELPVVERNDCQGFYYVKQRLGKFF 889
Artemia 231 QLDRTSFVCAGGEPGRDACQVIYSGTNVKSLTLDEASGFLPKSKIGDGGS 280
Pacifastacus 890 ILDK-SFMCAGGEENKDACE----------GDGGG 913
Artemia 281 PLVCETEPNR--FEVVGLVAWGIGCAEPGIPGVYVNVASYVDWIIKEIST 328
Pacifastacus 914 LLACQ-DPTTGDYVLVGLTAWGIGCGQKDVPGVYVDVQHFREWVNGIISK 962
Artemia 329 P--------------- 329
Pacifastacus 963 EPQQQQQQSAGGYSGK 978

dscam	
DNA amplified by primer	1 catagtccaagagtgaatgggggatgtaaaatcatattctgtcgg H S P R V N G G C K I I F C R 46 acacttggtctatttttcttcaacgttacaacaattactactctc T L G L F F F N V T T I T T L 91 gaatttctaactgaatatgtg 111 E F L T E Y V
ORF in the *Artemia* database	MLTKHGIGEIQFKGWNKRRMIWGIFGQNNEAYVGISYEVLQQIFFLDVQSSGSLVFPPFRAEDYRQEVHAQTYRCKGTN AVGTIVSRDVQVRAGNITISNIGNTIFRVFPASDIAVNVQVKFVCRRVSMHKNKRAFFSLGLVHNCSRGINCIQLDHRN FAKVNKATFSVPLRYGRQNGRIWGSKLPLLTKKLLLHSPRVNGGCKIIFCRTLGLFFFNVTTITTLEFLTEYVAPQLNDL RPSIIFSKRVYRYFEDCMFVSLVPLSSLMRLFHSEDGKKLITV
Sequence of *Pacifastacus leniusculus*	MGTNSMVWPLLMLLSAHVLHTVVCEESGPVIAEEPDNRVDFSNSTGANIHCSVRGHPKPT VVWVKADDGTAIGDVPGLRKVLSNGTLMFPPFRAEDYRQEVHAQVYRCQATNPHGTVHSR DVHVRAVVHQDYMTDVSLEYVIRGNSALLKCNIPSFVADFVSVQAWLTDNDQAYYPSDNY DGKYLVLPSGELHIRNVNSEDGFKSYKCRTVHRLTQETRLSATAGRLVISEPVGLSGPRF PNIDLSRTQARRAGSDFPLMCQAQAHPTPAFRWFKFSENGRKSPVELGDRVKQVGGTLII REAKVDDSGKYLCVVNNSVGGESVETVLTVTAPLSAQVEPNVQTVEFGRPATFTCTYKGN PVKSVTWLKDGVTINHKEAVMRIDTVSREDKGMYQCFVRNDQESAQATAELKLGGRFEPP QLTYTFETSTLQPGPSVFLKCVAAGNPTPEITWELDGTRLSNSERMQVGQYVTVNGEVVS HLNISAVHTNDGGLYACVASSTVGSVKHAARLNVYGLPYIRPMDKVAVVAGENMVVHCPV AGYPIDSIVWEKNGRMLPINRRQKTFTNGTPIVEAVERNSDQGRYTCVARNSQGYTARGD LDVQVMEKPKLLPFTFPVEVQAGQLLQVSCTLLSGDDPVTLQWYKDKQPLTSSSKFMINN VVSRMSQLILQNVGAEHSGSYACRAFNSVGEAVSSDILNVKVPPRWIVEPTDKAFALGSD ARLECKADGFPRPSLGWKKAAGRTPGDYRDLSVSNPNVKVTDDGTLQIGNIQKSHEGYYL CEANNGIGAGLSTVIYVRVQAPPQFKIQYRNQTASRGDDAVLECGAEGETPIGILWSKNK HSIEPSNEPRYTIREEMRGGGVHSSLSIKTTDRSDSAVYTCVATNAFGSADTNINLIIQE HPEQPNSLKVLDKSGRSVELSWTPPYNGNSPITRYIVEYKLSRRNWDSDGERMMVPGDQN MAAVLDLRPATTYHLRIVARNEIGDSDPSDTVTIITAEEAPSGAPRDLKVEAVDQSSLRV TWKPPVREEWNGDIQGYQVGYRLASSNNSYVYETVEFSKEMGKEHHLVISKLSVYTEYAV VVSAFNKIGQGPKTDEIRAYTAEGTPQQPPQDVTCTTLTSQTIRVSWSSPPLETVQGVIK GYKVIYGPSDTWYDEESKDTKITGSTETHLHGLQKYTNYSLQVLAFTSGGEGVRSQPIHC QTDQDIPESPTSVKALVMSADSILVSWLPPERPNGIITQYTVYYKEHGKSDSETVQQKLS PAQLSYEATGLKRRDDYVFWVTASTTVGEGEMSQLVHLKLSNKVPAKIASFDDEYVATYK EDVKLMCQAVGLPTPDIRWTIRGEPFTPNDRMRLLPEGSLLIREVSRDDAGEYTCHVENP YGQDTVTHTLLIQAPPHPPEITLQSTTTNSIEVKIKPSVIDDTTPIHGYTIFFKPEFSNW

	ESIQVSSSTRSYTLEGLWCGSRYQIYASAYNKIGTGESSEILNARTKGKKPEVPEVNRFV EVSSSSITLHLNAWLDGGCPMNYFVVEYKARHQAEWTMASNQVKPTGNYVIMELTPATWY NLRISAHNNAGSSVAEYECATLTLTGATLPPNVMEISPTWLPDWWPKWLDLNVLVPVIAT IVVIIVGIVVICVAVTRRKNGIENLRLREEVYQQYQYNASMPPPSTMDKRHPGFREELGY IPPPNRKLPPVPGSQYNTCDRIKRGGGPGRGTHATWDPRRPMYEELSLHPPPGRRIPPGG PHPTHGSQDTLRSGGDDEICPYATFHLLGFREEMDPQQAGNNFQTFPYQNGHGSQQNFVN SPASRSMPPSSTYYSTVPGDMTASRMSNSTFSPTYDDPARSDEESDQYGGSTYSGGGPYA RAIDSVSQSGTAKRLNGGPLPGAAPSGHFWSKGYAHKFLMNRGSTSGSAGHGSPEPPPPP PPRNGDLPLDSSGLGSSLNDSNNSTASNQFSEAECDHDLVQRNYGVKATKSTEEMRKLLD KNEAAAHIQNGGLRMVSDEMNV
Homology with ***Pacifastacus leniusculus***	*Artemia* 1 ----------MLTKHGIGEIQF--------- 12 ::.:....:.| *Pacifastacus* 1 MGTNSMVWPLLMLLSAHVLHTVVCEESGPVIAEEPDNRVDFSNSTGANIH 50 *Artemia* 13 ---KGWNKRRMIWGIFGQNNEAYVGISYEVLQQIFFLDVQSSGSLVFPPF 59 :|..|..:.:| :...:..| :| ....|:::. |:|:|:||| *Pacifastacus* 51 CSVRGHPKPTVVW-VKADDGTA-IG-DVPGLRKVL-----SNGTLMFPPF 92 *Artemia* 60 RAEDYRQEVHAQTYRCKGTNAVGTIVSRDVQVRA-------GNITISNI- 101 ||||||||||||.|||:.||..||:.||||.||| .:::::.: *Pacifastacus* 93 RAEDYRQEVHAQVYRCQATNPHGTVHSRDVHVRAVVHQDYMTDVSLEYVI 142 *Artemia* 102 -GNTIFRVFPASDIAVNVQVKFVCRRVSMH----KNKRAFF--------- 137 ||:.. :..|:. .||...||:. .|.:|:: *Pacifastacus* 143 RGNSAL-------LKCNIP-SFVADFVSVQAWLTDNDQAYYPSDNYDGKY 184 *Artemia* 138 ---SLGLVH----------NCSRGINCIQLDHRNFAKVNKATFSVPL 171 ..|.:| .| |.::.:..:.|..|...:...|.|: *Pacifastacus* 185 LVLPSGELHIRNVNSEDGFKSYKC-RTVHRLTQETRLSATAGRLVISEPV 233 *Artemia* 172 -----RY-----GRQNGRIWGSKLPLLTK---------- 190 |: .|...|..||..||:.: *Pacifastacus* 234 GLSGPRFPNIDLSRTQARRAGSDFPLMCQAQAHPTPAFRWFKFSENGRKS 283 *Artemia* 191 ----------KLLLHSPRVNGGCKIIFCRTLGLFFFNVTTITTLEF- 226 .|::...:|:...|.:.....:...:|.|:.|:.. *Pacifastacus* 284 PVELGDRVKQVGGTLIIREAKVDDSGKYLCVVNNSVGGESVETVLTVTAP 333 *Artemia* 227 LTEYVAPQLNDL---RPSIIFSKRVYRYFEDCMF-----VSLVPLSSLMR 268 |:..|.|.:..: ||:.. .|.: .|:..|...:. *Pacifastacus* 334 LSAQVEPNVQTVEFGRPATF----------TCTYKGNPVKSVTWLKDGVT 373

```
Artemia          269 LFHSEDGKKLITV-------------------------------------    281
                     :.|.|...::.||
Pacifastacus     374 INHKEAVMRIDTVSREDKGMYQCFVRNDQESAQATAELKLGGRFEPPQLT    423

Artemia          282 -------------------------------------------------    281

Pacifastacus     424 YTFETSTLQPGPSVFLKCVAAGNPTPEITWELDGTRLSNSERMQVGQYVT    473

Artemia          282 -------------------------------------------------    281

Pacifastacus     474 VNGEVVSHLNISAVHTNDGGLYACVASSTVGSVKHAARLNVYGLPYIRPM    523

Artemia          282 -------------------------------------------------    281

Pacifastacus     524 DKVAVVAGENMVVHCPVAGYPIDSIVWEKNGRMLPINRRQKTFTNGTPIV    573

Artemia          282 -------------------------------------------------    281

Pacifastacus     574 EAVERNSDQGRYTCVARNSQGYTARGDLDVQVMEKPKLLPFTFPVEVQAG    623

Artemia          282 -------------------------------------------------    281

Pacifastacus     624 QLLQVSCTLLSGDDPVTLQWYKDKQPLTSSSKFMINNVVSRMSQLILQNV    673

Artemia          282 -------------------------------------------------    281

Pacifastacus     674 GAEHSGSYACRAFNSVGEAVSSDILNVKVPPRWIVEPTDKAFALGSDARL    723

Artemia          282 -------------------------------------------------    281

Pacifastacus     724 ECKADGFPRPSLGWKKAAGRTPGDYRDLSVSNPNVKVTDDGTLQIGNIQK    773

Artemia          282 -------------------------------------------------    281

Pacifastacus     774 SHEGYYLCEANNGIGAGLSTVIYVRVQAPPQFKIQYRNQTASRGDDAVLE    823

Artemia          282 -------------------------------------------------    281

Pacifastacus     824 CGAEGETPIGILWSKNKHSIEPSNEPRYTIREEMRGGGVHSSLSIKTTDR    873

Artemia          282 -------------------------------------------------    281

Pacifastacus     874 SDSAVYTCVATNAFGSADTNINLIIQEHPEQPNSLKVLDKSGRSVELSWT    923
```

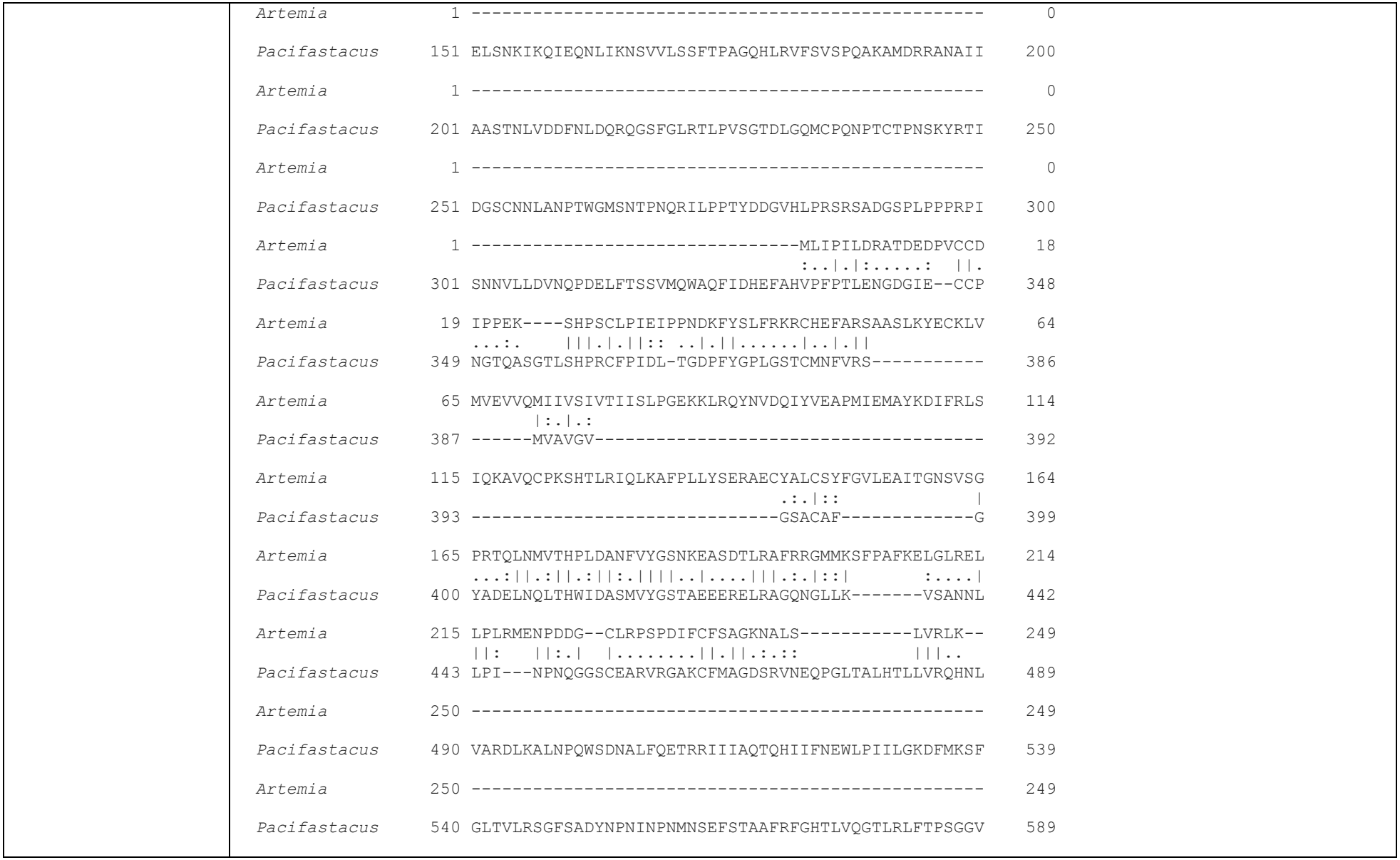
	Artemia 282 ---------- 281 *Pacifastacus* 924 PPYNGNSPITRYIVEYKLSRRNWDSDGERMMVPGDQNMAAVLDLRPATTY 973 *Artemia* 282 ---------- 281 *Pacifastacus* 974 HLRIVARNEIGDSDPSDTVTIITAEEAPSGAPRDLKVEAVDQSSLRVTWK 1023 *Artemia* 282 ---------- 281 *Pacifastacus* 1024 PPVREEWNGDIQGYQVGYRLASSNNSYVYETVEFSKEMGKEHHLVISKLS 1073 *Artemia* 282 ---------- 281 *Pacifastacus* 1074 VYTEYAVVVSAFNKIGQGPKTDEIRAYTAEGTPQQPPQDVTCTTLTSQTI 1123 *Artemia* 282 ---------- 281 *Pacifastacus* 1124 RVSWSSPPLETVQGVIKGYKVIYGPSDTWYDEESKDTKITGSTETHLHGL 1173 *Artemia* 282 ---------- 281 *Pacifastacus* 1174 QKYTNYSLQVLAFTSGGEGVRSQPIHCQTDQDIPESPTSVKALVMSADSI 1223 *Artemia* 282 ---------- 281 *Pacifastacus* 1224 LVSWLPPERPNGIITQYTVYYKEHGKSDSETVQQKLSPAQLSYEATGLKR 1273 *Artemia* 282 ---------- 281 *Pacifastacus* 1274 RDDYVFWVTASTTVGEGEMSQLVHLKLSNKVPAKIASFDDEYVATYKEDV 1323 *Artemia* 282 ---------- 281 *Pacifastacus* 1324 KLMCQAVGLPTPDIRWTIRGEPFTPNDRMRLLPEGSLLIREVSRDDAGEY 1373 *Artemia* 282 ---------- 281 *Pacifastacus* 1374 TCHVENPYGQDTVTHTLLIQAPPHPPEITLQSTTTNSIEVKIKPSVIDDT 1423 *Artemia* 282 ---------- 281 *Pacifastacus* 1424 TPIHGYTIFFKPEFSNWESIQVSSSTRSYTLEGLWCGSRYQIYASAYNKI 1473

	Artemia 282 ---------- 281 *Pacifastacus* 1474 GTGESSEILNARTKGKKPEVPEVNRFVEVSSSSITLHLNAWLDGGCPMNY 1523 *Artemia* 282 ---------- 281 *Pacifastacus* 1524 FVVEYKARHQAEWTMASNQVKPTGNYVIMELTPATWYNLRISAHNNAGSS 1573 *Artemia* 282 ---------- 281 *Pacifastacus* 1574 VAEYECATLTLTGATLPPNVMEISPTWLPDWWPKWLDLNVLVPVIATIVV 1623 *Artemia* 282 ---------- 281 *Pacifastacus* 1624 IIVGIVVICVAVTRRKNGIENLRLREEVYQQYQYNASMPPPSTMDKRHPG 1673 *Artemia* 282 ---------- 281 *Pacifastacus* 1674 FREELGYIPPPNRKLPPVPGSQYNTCDRIKRGGGPGRGTHATWDPRRPMY 1723 *Artemia* 282 ---------- 281 *Pacifastacus* 1724 EELSLHPPPGRRIPPGGPHPTHGSQDTLRSGGDDEICPYATFHLLGFREE 1773 *Artemia* 282 ---------- 281 *Pacifastacus* 1774 MDPQQAGNNFQTFPYQNGHGSQQNFVNSPASRSMPPSSTYYSTVPGDMTA 1823 *Artemia* 282 ---------- 281 *Pacifastacus* 1824 SRMSNSTFSPTYDDPARSDEESDQYGGSTYSGGGPYARAIDSVSQSGTAK 1873 *Artemia* 282 ---------- 281 *Pacifastacus* 1874 RLNGGPLPGAAPSGHFWSKGYAHKFLMNRGSTSGSAGHGSPEPPPPPPPR 1923 *Artemia* 282 ---------- 281 *Pacifastacus* 1924 NGDLPLDSSGLGSSLNDSNNSTASNQFSEAECDHDLVQRNYGVKATKSTE 1973 *Artemia* 282 ---------- 281 *Pacifastacus* 1974 EMRKLLDKNEAAAHIQNGGLRMVSDEMNV 2002

lgbp	
DNA amplified by primer	6 ctgatgctgaatggtgtcaatattggtgcagagcaagtttctcag L M L N G V N I G A E Q V S Q 51 acactacattggggtccacacttttttccttaaccaatatgagaaa T L H W G P H F F L N Q Y E K 96 acaagctggagcaagaattctgtccctggctatg 129 T S W S K N S V P G Y
ORF in the *Artemia* database	MLPRYQDYGQWPASGEIDIVESRGNSGLMLNGVNIGAEQVSQTLHWGPHFFLNQYEKTSWSKNSVP GYDSDFHIYGLEWTPEIIGKGMFAFDECKIRG
Sequence of *Pacifastacus leniusculus*	MLPRYQDYGQWPASGEIDIVESRGNSGLMLNGVNIGAEQVSQTLHWGPHFFLNQYE KTSWSKNSVPGYDSDFHIYGLEWTPEIIGKGMFAFDECKIRGMLPRYQDYGQWPAS GEIDIVESRGNSGLMLNGVNIGAEQVSQTLHWGPHFFLNQYEKTSWSKNSVPGYDS DFHIYGLEWTPEIIGKGMFAFDECKIRG
Homology with *Pacifastacus leniusculus*	*Artemia* 1 ---------- 0 *Pacifastacus* 1 MRALCFLLLACGALAVDVLDPGSCSSFPCLIFNDDFNDLNRNVWKPEVTM 50 *Artemia* 1 ---------- 0 *Pacifastacus* 51 SGGGNWEFQMYLNNPSLGYTRDSTLIIKPELTSKWYSEHFLFNDELNLGD 100 *Artemia* 1 ---------- 0 *Pacifastacus* 101 KCTDHRDYGCVRKGTSEHIINPIMSAKFTTHPSFAFRYGRVEVRAKMPRG 150 *Artemia* 1 ---------- 0 *Pacifastacus* 151 DWLWPAIWLMPKDSRYGGWPASGEIDIVESRGNNDYGNLGHQHAGSTLHW 200 *Artemia* 1 ----------MSRNLASQHLYSFSLISDY 19 |.....:...| :.|. *Pacifastacus* 201 GPNPQANMFLKTHKTYSANDGSFANNFHIWRMDWTRDNMKFY----VDDQ 246


Artemia 20 ISLTIDGVETGRVEPPANGFWEYGAFPAGQDNPWVGASKLAPFDQETSIM 69
Pacifastacus 247 LQLTVD---------PGSNFWDFGGLGNSQNNPWRDGSKMAPFDQK---- 283
Artemia 70 RNKSSQIQFSELNHPSQKLCQAQNLDIYILFTLIFGICHSNSFGLVVQSN 119
Pacifastacus 284 ---------- 283
Artemia 120 HQCDFVVSQLVVGYFSLLERLELFFYFIMNLAVGGTNSFFPDEAVNSGPP 169
Pacifastacus 284 ----------FYLILNLAVGGTNGYFPD-GVSSNPA 308
Artemia 170 KPWSNQSP----------QTIIFKMEYRNIRAIRE 194
Pacifastacus 309 KPWNNASPHASRDFWNARGSWLPSWEHGEGHISENAALKVDYVKVWKM-E 357
Artemia 195 TVEVGNEFKENAQHQCITMSLSGENRGLQYCIHLCIGGIRTKSDSYMHSY 244
Pacifastacus 358 SVEQ---------- 361
Artemia 245 QSVYDWYPIRTHISFCIRTKSGSKFA 270
Pacifastacus 362 ---------- 361

pero	
DNA amplified by primer	10 gctaccgatgaagatccagtatgctgtgacattccgccagaaaaa A T D E D P V C C D I P P E K 55 agtcaccatcttgtttaccaatcgaaattcctcccaatgataagt S H P S C L P I E I P P N D K 100 tttattcgctgttcaggaaaa F Y S L F R K
ORF in the *Artemia* database	MLIPILDRATDEDPVCCDIPPEKSHPSCLPIEIPPNDKFYSLFRKRCHEFARSAASLKYECKLVMVEVVQMIIVSIVTIIS LPGEKKLRQYNVDQIYVEAPMIEMAYKDIFRLSIQKAVQCPKSHTLRIQLKAFPLLYSERAECYALCSYFGVLEAITGNSV SGPRTQLNMVTHPLDANFVYGSNKEASDTLRAFRRGMMKSFPAFKELGLRELLPLRMENPDDGCLRPSPDIFCFSAGKNAL SLVRLK
Sequence of *Pacifastacus leniusculus*	MRQWSWLWVVVLGALVASQEPVDDSGEVVIDELPVARVVRQINFPGNQRRPPVGGNRPPTGGRLTGTPGG GGAGCNCVPAVVCASELDQLKDTCTTGGGAPGVCCPANALTAAAPAKGTADNRIFSGATVSVSLNPLDPK MMEASFKKGLELSNKIKQIEQNLIKNSVVLSSFTPAGQHLRVFSVSPQAKAMDRRANAIIAASTNLVDDF NLDQRQGSFGLRTLPVSGTDLGQMCPQNPTCTPNSKYRTIDGSCNNLANPTWGMSNTPNQRILPPTYDDG VHLPRSRSADGSPLPPPRPISNNVLLDVNQPDELFTSSVMQWAQFIDHEFAHVPFPTLENGDGIECCPNG TQASGTLSHPRCFPIDLTGDPFYGPLGSTCMNFVRSMVAVGVGSACAFGYADELNQLTHWIDASMVYGST AEEERELRAGQNGLLKVSANNLLPINPNQGGSCEARVRGAKCFMAGDSRVNEQPGLTALHTLLVRQHNLV ARDLKALNPQWSDNALFQETRRIIIAQTQHIIFNEWLPIILGKDFMKSFGLTVLRSGFSADYNPNINPNM NSEFSTAAFRFGHTLVQGTLRLFTPSGGVDTIRMRDHFNSPHLIETQGRLDDIVRSLTQLAIQKYDSFIT QDLSNHLFQTPRFNFGMDLMSLNIQRGRDHGIATYNSMRQVCGLPRARTFNDLTDQISPENVQKLARIYK NVDDIDLFVGGITENSVRGGLLGWTFLCIVGDQFARLKKGDRYFYDLGGQAGSFTEPQLQQIRASSWARI ICDTANVPAVQPLAFRQTNSRFNQPVPCNNPAIPRPNWAPWKGERPAV
Homology with Pacifastacus leniusculus	*Artemia* 1 ---------- 0 *Pacifastacus* 1 MRQWSWLWVVVLGALVASQEPVDDSGEVVIDELPVARVVRQINFPGNQRR 50 *Artemia* 1 ---------- 0 *Pacifastacus* 51 PPVGGNRPPTGGRLTGTPGGGGAGCNCVPAVVCASELDQLKDTCTTGGGA 100 *Artemia* 1 ---------- 0 *Pacifastacus* 101 PGVCCPANALTAAAPAKGTADNRIFSGATVSVSLNPLDPKMMEASFKKGL 150


Artemia 1 ---------- 0
Pacifastacus 151 ELSNKIKQIEQNLIKNSVVLSSFTPAGQHLRVFSVSPQAKAMDRRANAII 200
Artemia 1 ---------- 0
Pacifastacus 201 AASTNLVDDFNLDQRQGSFGLRTLPVSGTDLGQMCPQNPTCTPNSKYRTI 250
Artemia 1 ---------- 0
Pacifastacus 251 DGSCNNLANPTWGMSNTPNQRILPPTYDDGVHLPRSRSADGSPLPPPRPI 300
Artemia 1 ----------MLIPILDRATDEDPVCCD 18
Pacifastacus 301 SNNVLLDVNQPDELFTSSVMQWAQFIDHEFAHVPFPTLENGDGIE--CCP 348
Artemia 19 IPPEK----SHPSCLPIEIPPNDKFYSLFRKRCHEFARSAASLKYECKLV 64
Pacifastacus 349 NGTQASGTLSHPRCFPIDL-TGDPFYGPLGSTCMNFVRS---------- 386
Artemia 65 MVEVVQMIIVSIVTIISLPGEKKLRQYNVDQIYVEAPMIEMAYKDIFRLS 114
Pacifastacus 387 ------MVAVGV---------- 392
Artemia 115 IQKAVQCPKSHTLRIQLKAFPLLYSERAECYALCSYFGVLEAITGNSVSG 164
Pacifastacus 393 ----------GSACAF----------G 399
Artemia 165 PRTQLNMVTHPLDANFVYGSNKEASDTLRAFRRGMMKSFPAFKELGLREL 214
Pacifastacus 400 YADELNQLTHWIDASMVYGSTAEEERELRAGQNGLLK-------VSANNL 442
Artemia 215 LPLRMENPDDG--CLRPSPDIFCFSAGKNALS----------LVRLK-- 249
Pacifastacus 443 LPI---NPNQGGSCEARVRGAKCFMAGDSRVNEQPGLTALHTLLVRQHNL 489
Artemia 250 ---------- 249
Pacifastacus 490 VARDLKALNPQWSDNALFQETRRIIIAQTQHIIFNEWLPIILGKDFMKSF 539
Artemia 250 ---------- 249
Pacifastacus 540 GLTVLRSGFSADYNPNINPNMNSEFSTAAFRFGHTLVQGTLRLFTPSGGV 589

	Artemia	250	----------	249
	Pacifastacus	590	DTIRMRDHFNSPHLIETQGRLDDIVRSLTQLAIQKYDSFITQDLSNHLFQ	639
	Artemia	250	----------	249
	Pacifastacus	640	TPRFNFGMDLMSLNIQRGRDHGIATYNSMRQVCGLPRARTFNDLTDQISP	689
	Artemia	250	----------	249
	Pacifastacus	690	ENVQKLARIYKNVDDIDLFVGGITENSVRGGLLGWTFLCIVGDQFARLKK	739
	Artemia	250	----------	249
	Pacifastacus	740	GDRYFYDLGGQAGSFTEPQLQQIRASSWARIICDTANVPAVQPLAFRQTN	789
	Artemia	250	----------	249
	Pacifastacus	790	SRFNQPVPCNNPAIPRPNWAPWKGERPAV	818

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SUMMARY

SUMMARY

Sustainable growth for current aquaculture practices is mainly hampered by increasing incidences of disease and the correlated misuse of antibiotics. Vibriosis, caused by species belonging to the *Vibrio* genus, causes several billion US$ losses each year in the aquaculture sector. Antibiotic misuse has led to the development of antibiotic resistant strains, allergy and toxicity in human consumers. Novel preventive and curative approaches in aquaculture are needed, e.g. immunostimulants. β-glucans have proven potent, valuable and promising compounds for improving the immune status of aquatic organisms. The small-scale culturing system of *A. franciscana* under gnotobiotic conditions allows for a rapid screening of the effectiveness of these immunostimulants in the presence of a pathogen.

Chapter 1 is a literature review summarising the current state of aquaculture practices. Subsequently, the bottlenecks caused by disease and new possible ways of disease prevention, namely immunostimulants, are discussed. The immune responses known for crustaceans are layed out and finally, the relevance of gnotobiotic models is discussed and possible restrictions to their use as host-microbe research models are presented. The scientific aims of this PhD thesis are detailed in **Chapter 2**.

The *Vibrio* strain (*Vibrio campbellii* LMG 21363) currently used in the culturing system is not virulent enough to perform challenge tests on older animals. Therefore, a more virulent pathogen was needed for challenge tests, which was found in the newly identified *Vibrio* strain H6 (**Chapter 3**). H6 was obtained from a shrimp hatchery in Rio Grande do Norte (Natal, Brazil). To our knowledge, H6 is the most virulent strain described for the gnotobiotic *Artemia* model system thus far.

To prolong the gnotobiotic culture system, a poor performing feed, dead LVS3 cells, was replaced by the axenic microalgae *Tetraselmis suecica*. A comparative morphological study was performed between gnotobiotic and conventional *Artemia franciscana* to elucidate the effect of these axenic microalgae on the gastrointestinal tract. Results (**Chapter 4**) showed that axenic microalgae did not allow culturing *Artemia franciscana* under gnotobiotic

conditions until adult. Differences between gnotobiotic and conventional animals were minor at day 2. On the contrary, from day 4 onwards, gnotobiotic animals showed a deteriorated gastrointestinal tract and very low survival.

Even with this more virulent pathogen at hand, challenge tests with conventional animals showed that survival increased significantly after day 5/day 6 (**Chapter 5**). A well-known protective barrier is the acid mucins in the gastrointestinal tract. These mucins are produced by goblet cells, which only occur several days after hatching in several other animals. Morphological alterations in the gastrointestinal tract from day 2 until day 8 were identified in order to find an explanation in the increase in survival around day 5/day 6 after a *Vibrio* challenge. Indeed, the occurrence of goblet cells in the gastrointestinal tract could be observed at day 5 after hatching. The occurrence of these cells could provide an explanation for the changes in survival, however further research is warranted to elucidate other barriers involved in the protection of these animals against pathogens.

In the second part of this thesis, validation of the immunological response of *Artemia franciscana* was done after exposure to β-1.3/1.6-glucans (MacroGard) and a virulent pathogen. Based on results of previous chapters, it was decided to focus on the early stages of development (instar II nauplii) using the gnotobiotic culturing system, but with H6 as pathogen. The survival of the *Artemia* nauplii was observed after a 6 hour exposure period with the β-1.3/1.6-glucans followed by a challenge test. In this work, we demonstrate a significant increase in survival induced by β-1,3/1,6-glucans (**Chapter 6**). The pre-treatment of 6 h with β-1,3/1,6-glucans provided a significant protection against the pathogen H6, which lasted for at least 6 h.

The expression of eight immune-related genes of *Artemia franciscana* was investigated for the first time in this thesis (h*sp70, proPO, tgase, masq*, *esod*, *pero*, *lgbp* and *dscam*) (**Chapter 7**). Results indicate that β-1,3/1,6-glucans are promising as an alternative strategy in aquaculture to combat disease caused by vibrios. The studies on the effects of β-1,3/1,6-glucans on the gene expression in *Artemia franciscana* are still preliminary and remain to be further explored together with further validation of the newly identified genes.

Finally, the general discussion (**Chapter 8**) elaborates on the most important findings and discusses future prospects of this PhD research. The used gnotobiotic model proved to be a suitable system to evaluate the survival and immunological response of *Artemia franciscana* after exposure to β-1.3/1.6-glucans and a virulent pathogen. Nevertheless, precaution should be taken with older gnotobiotic animals.

In conclusion, the work presented in this thesis indicates that β-glucan particles stimulate the innate immune system in brine shrimp, thereby providing protection to the animals upon exposure to a pathogen. To evaluate the long-term effects an appropriate feed should be researched to grow the gnotobiotic *Artemia franciscana* until adult, without the observed consequences in this study on the alimentary tract.

SAMENVATTING

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Duurzame groei in de aquacultuur sector wordt vooral gehinderd door het hoge aantal gevallen van ziekte, die grote economische verliezen veroorzaken, en het gecorreleerde misbruik van antibiotica. Vibriosis, veroorzaakt door soorten die behoren tot het genus *Vibrio*, is verantwoordelijk voor het verlies van enkele miljarden US $ per jaar. Antibioticummisbruik heeft geleid tot de ontwikkeling van antibioticaresistente stammen, allergie en zelfs toxiciteit bij menselijke consumptie. Nieuwe preventieve en curatieve aanpakken zijn nodig, bijvoorbeeld immunostimulantia. β-glucanen zijn reeds krachtige en veelbelovende samenstellingen gebleken voor het verbeteren van de immuunstatus van gecultiveerde dieren. Gnotobiotische dieren, zoals *Artemia franciscana*, zijn zeer nuttig bij het bestuderen van gastheer-microbe interacties en voor een vlugge screening van deze immunostimulanten.

Hoofdstuk 1 geeft een overzicht van de huidige stand van zaken in de aquacultuur. Vervolgens worden de knelpunten ten gevolge van ziekte besproken, gevolgd door nieuwe mogelijkheden van ziektepreventie, namelijk immuunstimulantia. Immuunreacties van schaaldieren worden naar voor gebracht. Tot slot wordt de relevantie van gnotobiotische modellen besproken samen met mogelijke beperkingen met betrekking tot het gebruik ervan als gastheermicrobemodellen in wetenschappelijk onderzoek. De wetenschappelijke doelstellingen van dit proefschrift zijn samengevat in **Hoofdstuk 2**.

De *Vibrio* stam, *Vibrio campbellii* LMG 21363, die momenteel wordt gebruikt in het gnotobiotisch *Artemia*systeem is niet virulent genoeg om mortaliteit te induceren bij oudere dieren. Er is een meer virulente pathogeen nodig om de langetermijneffecten van β-1,3/1,6-glucanen op oudere *Artemia franciscana* te onderzoeken. Die is gevonden in de nieuw geïdentificeerde *Vibrio* stam H6 (**Hoofdstuk 3**). H6 werd verkregen van een garnaalboerderij in Rio Grande do Norte, Natal, Brazilië. Voor zover bekend is, is H6 de meest virulente stam tot dusver beschreven voor het gnotobiotische *Artemia*modelsysteem.

Om het gnotobiotisch cultuursysteem te kunnen verlengen werd het huidige voeder, dode LVS3 cellen, vervangen door de axenische microalge *Tetraselmis suecica*. Een vergelijkende

morfologische studie tussen gnotobiotische en conventionele *Artemia franciscana*dieren werd uitgevoerd om het effect van deze axenische microalgen op de ontwikkeling van het maagdarmkanaal op te helderen. Uit resultaten (**Hoofdstuk 4**) bleek dat deze axenische microalgen het niet toestonden om *Artemia franciscana* onder gnotobiotische omstandigheden tot volwassen dieren op te kweken. De verschillen tussen gnotobiotische en conventionele dieren waren minimaal op dag 2. Daarentegen, vanaf dag 4 vertoonden de gnotobiotische dieren een achteruitgang in de toestand van het maagdarmkanaal samen met een zeer lage overleving.

Zelfs met een virulentere pathogeen bleek de mortaliteitstest met oudere conventionele dieren niet mogelijk, overleving nam aanzienlijk toe na dag 5 en 6 (**Hoofdstuk 5**). De mucuslaag in het maagdarmkanaal is een beschermende laag tegen indringende pathogenen. De zuren worden geproduceerd door slijmbekercellen, die bij andere dieren slechts enkele dagen later in de ontwikkeling gevormd worden. Morfologische veranderingen van het maagdarmkanaal werden daarom vastgelegd van dag 2 tot en met dag 8 om een mogelijke verklaring te vinden voor de toename in overleving. Slijmbekercellen werden inderdaad ook bij *Artemia* waargenomen op dag 5 in het maagdarmkanaal. Het voorkomen van deze cellen zou een verklaring kunnen verschaffen voor de veranderingen in overleving. Verder onderzoek wordt echter aangeraden om de bescherming van deze dieren tegen pathogenen op te helderen.

In het tweede deel van dit proefschrift, werd de immunologische respons van *Artemia franciscana* gevalideerd na blootstelling aan β-1,3/1,6-glucanen (MacroGard) en een virulente pathogeen. Gebaseerd op resultaten uit de voorgaande hoofdstukken werd besloten zich toch te richten op de vroege stadia van ontwikkeling (instar II nauplii) met het gnotobiotische systeem, maar met H6 als pathogeen. De overleving van de *Artemia* nauplii werd geteld na een blootstelling van 6 uur aan β-1,3/1,6-glucanen, gevolgd door een mortaliteitstest met H6. In dit werk tonen we een significante toename in overleving aan, na een blootstelling van 6 uur aan β-1,3-glucanen (**Hoofdstuk 6**). De voorbehandeling met β-1,3-glucanen leverde een significante bescherming op tegen de pathogeen H6, die minstens 6 uur aanhield.

De expressie van acht immuungerelateerde genen van *Artemia franciscana* werd voor de eerste keer onderzocht in dit doctoraatswerk (*hsp70, proPO, tgase, masq, esod, pero, lgbp* en

dscam) (**Hoofdstuk 7**). Resultaten tonen aan dat β-1,3-glucanen veelbelovend zijn als alternatieve strategie voor de bestrijding van ziektes veroorzaakt door vibrios in de aquacultuur sector. Het onderzoek naar de effecten van β-1,3-glucanen op de genexpressie in *Artemia franciscana* staat voorlopig wel nog in zijn kinderschoenen en moet nog verder onderzocht worden.

Ten slotte wordt er in de algemene discussie (**Hoofdstuk 8**) dieper ingegaan op de belangrijkste bevindingen van dit doctoraat en worden er enkele perspectieven voorgesteld voor onderzoek in de toekomst. Het gebruikte gnotobiotisch model blijkt een geschikt systeem te zijn om de overleving en immunologische respons van *Artemia franciscana* te evalueren na blootstelling aan β-1,3/1,6-glucanen en een ziekteverwekker. Toch moeten de nodige voorzorgen genomen worden met oudere gnotobiotische dieren.

Het werk gepresenteerd in dit proefschrift geeft aan dat β-glucanen het aangeboren immuunsysteem van *Artemia franciscana* stimuleren, waardoor de dieren beschermd worden bij blootstelling aan een pathogeen. Om de lange-termijneffecten van deze glucanen te onderzoeken moet echter een passend voeder gevonden worden om de gnotobiotische *Artemia franciscana* tot volwassen dieren op te kweken zonder de waarnemingen gemaakt in deze studie met betrekking tot het maagdarmkanaal.

CURRICULUM VITAE

Sofie VANMAELE

sofie.vanmaele.sv@gmail.com

Date of birth: 05/11/1986, Belgium

WORK EXPERIENCE

RESEARCH ACTIVITY

PhD in Applied Bioscience

Immunostimulation in crustaceans: A gnotobiotic *Artemia* model system

Expected in 2014. With Bossier P., Cox E., Defoirdt T & Van Den Broeck W.

Ghent University (Ghent, Belgium), Department of Bioengineering, Faculty of Animal Production (Ghent, Belgium). Laboratory of Aquaculture & *Artemia* Reference Center.

Focus: Immunostimulation in the crustacean *Artemia franciscana*.
Techniques: Gnotobiotic model systems. Challenge tests. Microbiological techniques for characterization of pathogens. Gene expression (RT-PCR, Western blot, Bioinformatics). Microscopy and staining techniques (fluoromicroscopy, transmission electron microscopy). *Artemia* and microalgae cultures (axenic and conventional).

Master in Marine Science

Rooting biodiversity studies in "good taxonomy": are historical name-bearing types a boost or an impediment?

2010. With Bouchet P. & Puillandre N.

Museum National d'Histoire Naturelle (Paris, France). Department of Systematics & Evolution, Group of Malacology.

Focus: Comparative study between conventional taxonomy and DNA based phylogeny for the Triphoridae family.
Techniques: Microscopical manipulation and morphological study of mollusks. Phylogenetic analysis. Gene expression. Microphotography.

NATIONAL AND INTERNATIONAL PROJECTS

AQUA-TNET project. European thematic network in aquaculture, fisheries and aquatic resources management.

- 2012: Stakeholder think tank meeting in Brussels, Belgium
- 2011: Annual meeting in Faro, Portugal

FEAP project. Federation of European Aquaculture Producers.

- 2012: Annual meeting "Aquaculture in motion" in Brussels, Belgium

Host microbial interactions in aquatic production. European project funded by BOF (Belgian Special Research Fund), framework of my PhD.

CONFERENCES & BOARD MEMBERSHIP

European Aquaculture Society (Student Group)
- 2011-2013: Board member and treasurer

Larvi conference. Fish & Shellfish Larviculture Symposium.
- 2013: Coordination of the student volunteers

Aquaculture Europe Conference. Organization of 5 student events.
- 2012: Prague, Czech Republic
- 2011: Rhodes, Greece

COMMUNICATIONS

Peer-reviewed articles

Vanmaele S., Defoirdt T., Cleenwerck I., De Vos P., Bossier P. (2015). Characterisation of the virulence of Vibrios isolated from a shrimp hatchery, in vitro and in vivo in a gnotobiotic brine shrimp (*Artemia franciscana*) model system. Aquaculture, 28-32.

Vanmaele S., Defoirdt T., Bossier P. (2013) A gnotobiotic model system: the case of *Artemia franciscana*. Communications in agricultural and applied biological sciences 78(4): 465-8.

Oral communications

Vanmaele S., Defoirdt T., Bossier P. (2014) A new & more virulent Vibrio in a gnotobiotic *Artemia* challenge test. Vibrio 2014, Edinburgh, Scotland.

Vanmaele S., Defoirdt T., Bossier P. (2012) Immunostimulation through the eyes of gnotobiotic *Artemia franciscana*. Aquaculture Europe 2012, Prague, Czech Republic.

Vanmaele S. & Bossier P. (2012) Aquaculture & immunostimulation through the eyes of *Artemia franciscana*. 12th Young Marine Scientists' Day, Bruges, Belgium.

Poster presentations

Vanmaele S., Defoirdt T., Bossier P. (2013) A gnotobiotic model system: the case of *Artemia* franciscana. Larvi 2013, Ghent, Belgium.

Vanmaele S., Defoirdt T., Bossier P. (2013) Immunostimulation through the eyes of gnotobiotic *Artemia franciscana*. ISFSI 2013, Vigo, Spain.

Vanmaele S. & Allewaert C. (2010) Discovering marine biodiversity through integrative taxonomy - 2 case studies: the Triphoridae, a family of micromolluscs & the Mediterranean sponge genus Hexadella. Benelux Congress of Zoology 2010, Ghent, Belgium.

Vanmaele S., Puillandre N., Bouchet P. (2010) A molecular approach to complement traditional morphospecies delimitation: the case of the Triphoridae. European Consortium of Life 2010, Braga, Portugal.

EDUCATION

Master

M.Sc. Marine Biodiversity & Conservation. Erasmus Mundus Program (2010).
Ghent University (Ghent, Belgium) & Université Pierre & Marie Curie (Paris, France).
Magna cum Laude

THESIS:

Vanmaele S., Bouchet P. (2010) Rooting biodiversity studies in "good taxonomy": are historical name-bearing types a boost or an impediment? Master thesis. Université Pierre et Marie Curie & Museum National d'Histoire Naturelle (Paris, France).

Bachelor

B.Sc. Biology (2008), preceded by 2 years of B.Sc. Biochemistry & Biotechnology.
Ghent University (Ghent, Belgium).
Magna cum Laude

THESIS:

Vanmaele S., Vanreusel A. (2008) Meiofauna of hydrothermal vents in the deep sea. Bachelor thesis. Ghent University (Ghent, Belgium).

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